TISSUE INJURY BY VOLATILE AND GASEOUS ANAESTHETICS

by

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INTRODUCTION.

In our experiments with Holden (1938) on tissue injury by radiant energy, we used the perfused lungs of dogs anaesthetized with morphine and chloroform. In control experiments, though no significant output of histamine occurred, traces were found in the perfusate after 3 to 4½ hours' perfusion. It seemed possible that the short anaesthesia with chloroform might be responsible for this small effect. Bartosch (1936) did not observe any liberation of histamine from the isolated perfused lungs of guinea-pigs after the injection of 0·02 c.c. of chloroform, ether or ethylchloride into the tracheal cannula, though benzol, toluol and other hydrocarbons in this dose caused typical "lung shock" and the liberation of histamine. Garan (1938) confirmed the positive observations using various concentrations of the vapour of the hydrocarbons in the air with which the isolated lungs were ventilated.

The lung of the dog appears to be more sensitive than that of the guinea-pig and is suitable for comparison of the injurious effects of volatile and gaseous anaesthetics, provided that the preparation can be made under an anaesthetic which does not cause obvious injury to the lung tissue.

METHODS.

Our experiments were made on the artificially perfused left lungs of dogs. The dogs, weighing from 7 to 12 kg. received a subcutaneous injection of morphine hydrochloride (12 mg. per kg.) and were anaesthetised by the injection of chloralose (7·5 c.c. of 1 p.c. solution per kg.) into the external saphenous vein. The perfused left lung was set up on a paraffin block as described by Kellaway, Holden and Trethewie (1938). The lung was ventilated at 5·5 litres per minute (500 c.c. per stroke). After the blood had been washed out from the organ the perfusion rate was reduced to 1·0 to 1·5 c.c. per minute.

In the experiments with the volatile anaesthetics (chloroform, ethyl chloride and ether), the anaesthetic was placed in the Wolff's bottle (Fig. 1). The bottle with the anaesthetic it contained

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was weighed before and after the administration and the amount of anaesthetic volatilized was measured, from which the volume per cent. was calculated. By means of the clamps shown in the figure it was possible to put the anaesthetic bottle into the circuit or exclude it without altering the ventilation of the lung, and also to administer the anaesthetic in various concentrations determined by trial before each experiment. The clamps C and D on the inlet and outlet to the bottle were kept closed until it was desired to administer the anaesthetic, the clamp E remaining open and the screw clamp F having been adjusted for the percentage of anaesthetic it was desired to deliver. To administer the anaesthetic, clamp E was closed and C and D were opened. The inlet (A) and outlet (B) tubes in the bottle were moved during the experiment so as to keep them at fixed distances above the surface of the anaesthetic. For ether the Wolff's bottle was

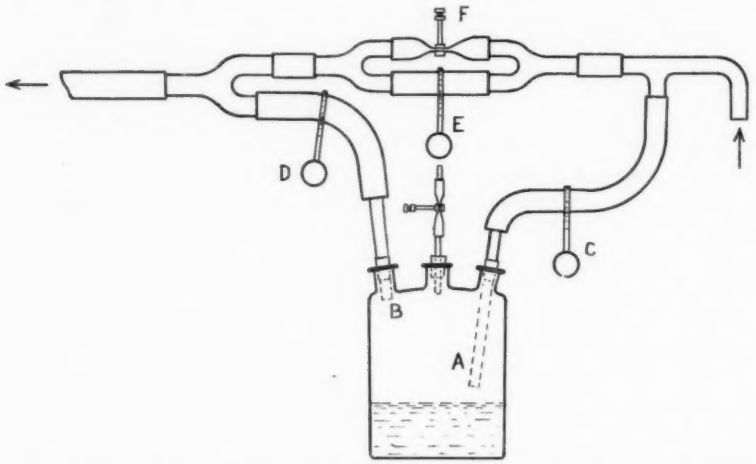

Fig. 1. Diagram showing arrangement of Wolff's bottle containing liquid anaesthetic, with by-pass. Arrows indicate direction of flow of air from the pump to the lung. Details in text.

immersed in a bath kept at 40°C. by a steady inflow of warm water and for ethyl chloride the bath was kept at 25°C. To obtain the highest concentrations used the air was bubbled through the anaesthetic and B was withdrawn as far as possible. In these experiments the air charged with anaesthetic on its way to the lung was bubbled through a wash bottle containing water at room temperature (19–21°C.). This was done so as to avoid the carriage of droplets of liquid anaesthetic and the condensation of anaesthetic vapour in the lung.

The gaseous anaesthetics (ethylene and nitrous oxide) were administered either pure or with various concentrations of oxygen by feeding the gases from an Austox anaesthetic machine to the inlet of the pump. In these and in the chloroform experiments the air passed through a spiral immersed in water at 40°C. between the anaesthetic bottle and the tracheal cannula.

Histamine was estimated on the isolated jejunum of the guinea-pig and is expressed as dihydrochloride. By using test doses of 1·0 c.c. of perfusate it could be determined in concentrations as low as 1: 50 millions. In the experiments with higher concentrations of chloroform and ether in which large amounts of histamine were liberated, the estimations on the guinea-pig's jejunum were checked by assay on the blood pressure of the cat before and after atropinization. The histamine in the lung was estimated in the right lung before and in the left lung after the

experiment. The pieces of lung used for extraction were pressed between filter papers to remove surface and oedema fluid before being weighed and ground up. Oedema in the left lung was estimated by weighing it before and after the experiment, the latter weighing being made after draining the lung with its bronchi dependent for ten minutes.

The perfusate was also tested for the presence of the substance described by Feldberg and Kellaway (1938) which, after a latent interval, causes contraction of the gut followed by slow relaxation and by characteristic after-changes in the excitability of the muscle. This substance we have called S.R.S. (slow reacting substance). Since there is much individual variation in the sensitivity of the guinea-pig's jejunum to S.R.S. we tested each preparation with a dose of a standard prepared by Mr. H. F. Holden by the action of snake venom on egg yolk.

In some experiments the coagulable protein in the perfusate was estimated roughly by Esbach's method.

RESULTS.

The isolated lung of the dog anaesthetized with morphia and chloralose can be perfused for 4½ to 5 hours without the appearance of detectable amounts of histamine or S.R.S. in the perfusate. At the end of this time the drainage fluid usually contains some histamine (nil to 1 : 20 millions) and some degree of oedema of the lung (about 30 p.c.) is found. In some experiments gaseous anaesthetics were administered pure or with amounts of oxygen less than that contained in atmospheric air, and since the organ was perfused with Tyrode solution, which cannot be relied upon for the transport of oxygen, a further control was desirable. In these control experiments the lung was ventilated for two hours with nitrogen, and no histamine appeared in the perfusate either during its administration or in the succeeding 2 to 2½ hours. The drainage fluid contained histamine in amounts ranging from 1 : 40 to 1 : 10 millions, and the oedema present varied from 30 to 40 p.c.

Ether.

Ether in high concentrations (19–44 p.c.) causes the liberation of histamine and S.R.S. from the isolated perfused lung of the dog. With moderate concentrations (10–16 p.c.) no histamine appears in the perfusate; S.R.S. is liberated, at the lower limit (10 p.c.) in small amounts and for a short period only, but with higher concentrations in considerable amounts. Ether in concentrations less than 10 p.c. is without obvious effect.

The effect of a high concentration is illustrated by an experiment in which 44 p.c. ether was administered for half-an-hour to a lung weighing 41 gm. During perfusion for an hour before the anaesthetic was administered the perfusate was free from detectable histamine and S.R.S. The output of histamine during the anaesthetic and in three-and-a-half hours' subsequent perfusion is shown in Fig. 2 (continuous line). The drainage fluid collected in ten minutes contained 1 : 2·5 millions (6γ), making a total output in four hours from the commencement of the anaesthetic of 312γ histamine. The lung now weighed 53 gm., about 30 p.c. increase in weight being caused by oedema. The histamine estimated in the right

lung was 60γ per gm., and in the left lung at the end of the experiment 33γ per gm. The early samples of perfusate contained so much histamine that the estimation of S.R.S. was difficult, but it was present in the later samples, and in the last 1·0 c.c. contained an amount equivalent to 2 mg. of the standard preparation. The perfusate before the anaesthetic was administered contained traces of coagulable protein (less than 0·4 mg. per minute). During the half-hour of administration this increased to 2·0 mg. per minute and fell during the last hour of perfusion to 0·6 mg. per minute.

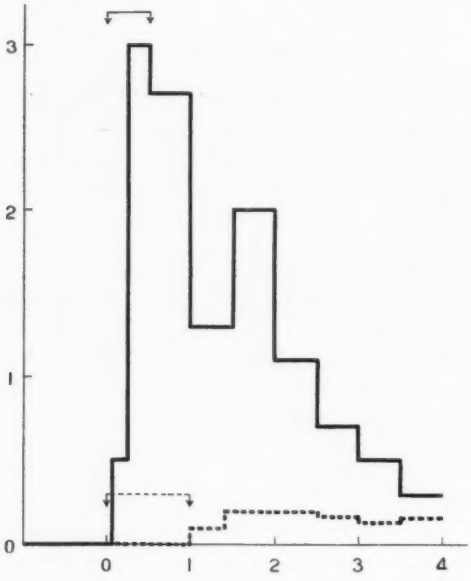

Fig. 2. Output of histamine from the isolated perfused lungs of two dogs ventilated with ether; continuous line 44 p.c., interrupted line 19 p.c. ether. Period of administration indicated by arrows. Ordinates histamine in γ per min.; abscissae time in hours.

The effect of about half this concentration is illustrated by an experiment in which 19 p.c. ether was administered for one hour to a lung weighing 56·5 gm. The perfusate before and during the administration contained no detectable amounts of histamine. The output of histamine during the subsequent perfusion is shown in Fig. 2 (interrupted line). The drainage fluid in ten minutes contained 1 : 4 millions histamine (3γ). The total output of histamine was 32γ. The lung at the end of the experiment weighed 72 gm., an increase of about 27 p.c. The histamine content of the right lung was 90γ per gm., and that of the left lung after the experiment 100γ per gm. No slow reacting substance was present in the

perfusate before administration, but traces appeared between 5 and 15 minutes after the anaesthetic was commenced, and the sample collected during the last half-hour of administration contained a moderate amount. In the succeeding samples and in the drainage fluid much more S.R.S. was present.

The effect of a smaller dose is illustrated by an experiment in which ether 16 p.c. was administered for one hour to a lung weighing 45 gm. The perfusate before and during the administration and the ensuing three hours' perfusion con-

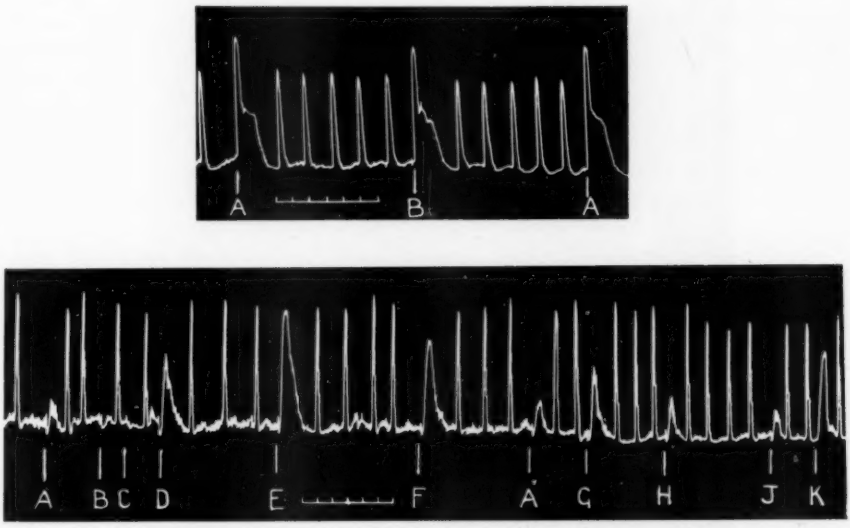

Fig. 3. Upper panel—responses of guinea-pig's jejunum to extracts of lung—A control, B incubated with ether before extraction. Lower panel—responses of jejunum from another guinea-pig to perfusates and drainage fluid from a dog's lung ventilated with ether (16 p.c.). The unlettered contractions are to 0·1γ histamine. Time in minutes. Details in text.

tained no detectable histamine. The drainage fluid contained about 1 : 30 millions histamine and the lung weighed 65 gm., its weight being increased by oedema to the extent of 42 p.c. The right lung and the left lung after perfusion both contained 80γ histamine per gm. The perfusate before and during the administration of the anaesthetic contained no appreciable S.R.S., but the output which was observed in the succeeding samples is illustrated in Fig. 3 (lower panel) which shows the reaction of the isolated jejunum of a guinea-pig to doses of 1·0 c.c. of a number of the samples of perfusate. At A the sample before the administration and at B and C the last two samples during the administration of the anaesthetic contained no significant amount of S.R.S. The contractions at D, E, F, G, H and J

all had a latency of 10 seconds and were caused by the successive half-hour samples of perfusate after the end of the administration of the anaesthetic. They exhibit an increase to a maximum at E, falling off to negligible responses at H and J. The contraction caused by the drainage fluid at K was immediate, and was partly caused by the histamine present in it. There was no increase in the coagulable protein (measured by the Esbach method) in the perfusate.

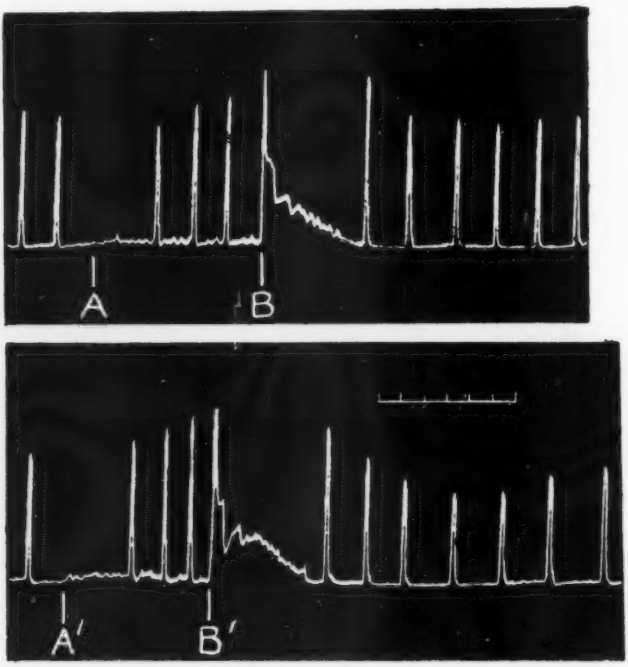

Fig. 4. Responses of the isolated jejunum of a guinea-pig to sediment and supernatant of an extract of dessicated dog's lung. At A, boiled and at A′, unboiled sediment, at B, boiled and at B′, unboiled supernatant equivalent to 5 mg. tissue. The unlettered contractions are to 0·1γ histamine. Time in minutes. Details in text.

In a number of experiments in which ether was given in concentrations between 12 and 18 p.c. we observed an output of S.R.S. in the perfusate, and it seemed important to determine whether this was liberated from the slightly injured tissues or whether it was actually formed by the action of ether on the tissues. To clear up this point it was necessary to use a method of extraction which would ensure that all the substance with a slow reacting action which was present in the tissues was extracted. This we did by thoroughly drying the ground tissue over sulphuric

acid *in vacuo* and then making saline extracts. These were centrifuged and the residues again extracted. To show that all the histamine was extracted in this manner the sediment was tested after boiling, which did not cause any further release of histamine. In Fig. 4, at A and A′, the boiled and unboiled sediment was tested and shown to contain neither histamine nor S.R.S., and at B and B′ the supernatant fluid, tested boiled and unboiled, contained equal amounts of histamine. A small amount of S.R.S. was apparently lost by boiling.

Two pieces of the right lung of a dog which contained 45γ histamine per gm. were ground up and incubated for half-an-hour at 37° C., one with and one without ether (0·4 c.c. ether to 0·4 gm. lung). The saline extracts were made after drying the control and experimental samples for 48 hours. In Fig. 3 (upper panel) the results of tests of extracts equivalent to 5 mg. of lung (control at A and incubated with ether at B) show that there is no appreciable difference in the amount of S.R.S. present. It is likely therefore that the S.R.S. observed in the perfusates after administration of ether is liberated from the tissues and not formed there in the course of the cell injury.

Chloroform.

Chloroform in high concentrations (4·4 p.c. to 8 p.c.) causes severe injury to the perfused lung, and large quantities of histamine and coagulable protein appear in the perfusate. S.R.S. is found in moderate amounts rather irregularly. Administered for one hour in concentrations ranging from 2·2 to 3·8 p.c. chloroform does not cause any detectable output of histamine, and S.R.S. is not found in the perfusate during subsequent perfusion for three hours. The drainage fluid at the end of this time sometimes contains small amounts of histamine.

In order to gain an approximate idea of the threshold of concentration at which injury to the lung took place several experiments were made in which increasing concentrations of chloroform were administered to the same lung each for half-an-hour until histamine appeared in the perfusate. For example, chloroform was administered to a perfused lung weighing 38 gm. for successive half-hour periods in concentrations of 1·8 p.c., 2·3 p.c., 2·7 p.c. and 4·6 p.c. No histamine appeared in the perfusate until the sample collected during the last half of the period during which chloroform 4·6 p.c. was given. This sample contained 1 : 10 millions (1γ) of histamine, and the ensuing half-hour samples contained 1 : 7 millions (7γ), 1 : 4 millions (11γ), 1 : 2·8 millions (14γ), 1 : 3 millions (14γ), 1 : 3 millions (14γ), and 1 : 4 millions (10γ). The drainage fluid contained 1 : 3·5 millions (4γ). The total output of histamine in three hours' perfusion after the end of the administration of anaesthetic was 75γ histamine. The lung now weighed 51 gm., having increased 34 p.c. by oedema. The right lung and a sample of the left lung both contained 27γ histamine per gm. At no stage during the experiment was any S.R.S. found in the perfusate.

A number of experiments were made in which concentrations of chloroform ranging from 2·2 to 4·4 p.c. were administered for one hour and the perfusate tested for the succeeding three hours. In those in which concentrations of 2·2, 3·1, 3·2 and 3·8 p.c. were tested the left lungs weighed 36, 27, 42 and 34 gm., and the corresponding right lungs contained 33, 63, 17 and 8γ per gm. histamine respectively. In none of them was any detectable histamine or S.R.S. found in the perfusate, but the drainage fluid at the end of the experiment in each case contained a small amount of histamine: 1 : 40 millions, 1 : 25 millions, less than 1 : 50 millions and a trace. The content of histamine in samples of the left lungs after

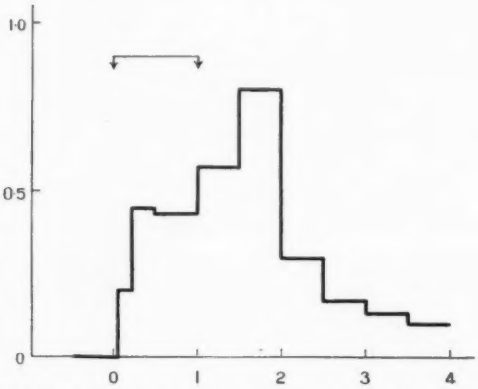

Fig. 5. Output of histamine from the isolated lung of a dog. Ordinates, histamine in γ per min.: abscissae—time in hours. Between the arrows chloroform 4·4 p.c. was administered.

the experiment were 37, 67, 20 and 8γ per gm. respectively. Chloroform in the concentration in which it is used for anaesthesia and even in somewhat higher concentrations has no appreciable injurious effect upon the isolated lung of the dog.

With a concentration of 4·4 p.c. there is a moderately severe degree of injury to the perfused lung. A lung weighing 31 gm. was ventilated with air containing 4·4 p.c. chloroform for one hour. The first five-minute sample of perfusate obtained during the administration, like those before it, contained no detectable histamine. The output of histamine in the succeeding samples of perfusate is shown in Fig. 5. The drainage fluid contained 1 : 5 millions (2γ) histamine. The total output of histamine was 86γ. The lung now weighed 35 gm., showing an increase due to oedema of 13 p.c. The right lung contained 37γ histamine per gm. and the left lung 33γ. Only traces of S.R.S. were found in the perfusate after the administration of chloroform.

With higher concentrations of chloroform much larger amounts of histamine were set free into the perfusate, and in these, as in the experiments with high concentrations of ether, we took the opportunity of titrating the histamine on the cat's

blood pressure as well as upon the isolated jejunum of the guinea-pig. Such a comparison is illustrated in Fig. 6. The left-hand panel shows the fall of blood pressure in a cat caused by the intravenous injection of 0·4 c.c. of a sample from a perfused lung ventilated with chloroform 7 p.c. (B) and each side of this, responses to 0·4 c.c. of histamine 1 : 200,000 (H) and 1 : 150,000 (H′). A dose of 1·0 c.c. of a sample of perfusate before the administration of chloroform (A) was without depressant action. The right-hand panel shows responses to the same test doses after

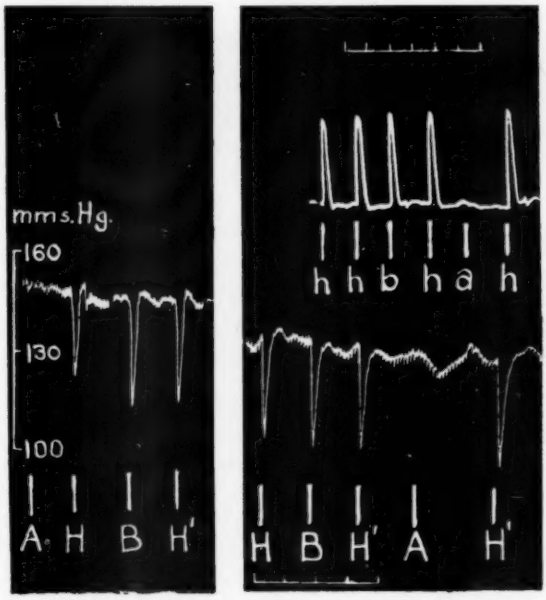

Fig. 6. Titration of histamine in a sample of perfusate from a dog's lung ventilated with chloroform, on the carotid blood pressure of a cat weighing 2·6 kg. under chloralose anaesthesia and on the isolated jejunum of a guinea-pig. Time in minutes. Details in text.

atropinization. The inset in the right-hand panel shows the response of the isolated jejunum of the guinea-pig to 0·1γ histamine at h and to 0·15 c.c. of the sample (ten times diluted) after chloroform at b. A dose of 0·2 c.c. of the sample before administration was without effect at a. The sample of perfusate obtained after chloroform administration is by both tests equivalent to 1 : 150,000 histamine.

Ethyl Chloride.

Ethyl chloride administered in a concentration of about 40 p.c. for half-an-hour causes some degree of injury to the perfused lung with the liberation into the

perfusate of histamine and S.R.S. in moderate amounts. In half this concentration it is without effect, and in intermediate percentages it causes a small output of histamine and a moderate output of S.R.S.

In a preliminary ranging experiment a lung weighing 20 gm. was perfused and ventilated with ethyl chloride 22 p.c. for half-an-hour. Neither histamine nor S.R.S. was found in the perfusate during this period, nor in the succeeding hour. Ethyl chloride 37 p.c. was now given for half-an-hour, and during the last 20 minutes of the administration histamine 1 : 5 millions appeared in the perfusate. During the next two half-hour periods it was present in concentrations of 1 : 10 millions and 1 : 17 millions. The next half-hour sample was free from detectable histamine, and the drainage fluid contained less than 1 : 20 millions. The total output of histamine was less than 3γ. S.R.S. was present in those samples which contained histamine and in the drainage fluid. The lung now weighed 25 gm., having increased 25 p.c. by oedema. The right lung contained 13γ per gm. histamine, and 8γ per gm. was estimated in the left lung after perfusion.

The effect of a high percentage of ethyl chloride was seen in a lung weighing 27 gm., which was ventilated with this anaesthetic in a concentration of 44 p.c. for half-an-hour. The samples collected before and during the first five minutes of the administration of ethyl chloride were free from detectable histamine and S.R.S. A sample collected during the next ten minutes contained histamine 1 : 11 millions (1γ) and that in the last fifteen minutes of anaesthesia contained 1 : 7 millions (3γ). In five succeeding half-hour samples the concentrations of histamine found were 1 : 8 millions (5γ), 1 : 13 millions (3γ), 1 : 20 millions (1γ), 1 : 25 millions (1·5γ), and 1 : 40 millions (1γ). The drainage fluid contained 1 : 25 millions histamine (0·5γ), and the total output was 16γ. The lung now weighed 45 gm., having increased 66 p.c. from oedema. The right lung and the left lung after perfusion both contained 16γ per gm. histamine. S.R.S. was present in all the samples of perfusate which contained histamine, except the last and the drainage fluid, from which it was absent. Fig. 7 illustrates the output of histamine and of S.R.S. in this experiment. It shows the responses to 1 c.c. of samples of perfusate left in contact with the gut for 10 seconds before washing out. At B, the sample collected before, at D, that collected during the last 15 minutes of administration, and at C, A and E, samples collected during the first half-hour, between half-an-hour and one hour, and between two and two-and-a-half hours after the anaesthetic had been given, were added to the bath. At F the drainage fluid was tested. The maximum response to S.R.S. was obtained at C during the first half-hour after administration of ethyl chloride. It will be noted that owing to the presence of histamine the responses at A, C, D, E and F were immediate and that S.R.S. in the samples tested at A, C and D is shown by delayed relaxation after washing out.

A lung weighing 29 gm. was ventilated with 27 p.c. ethyl chloride for half-an-hour. Neither histamine nor S.R.S. appeared in the perfusate before or during

this treatment. During three-and-a-half hours' further perfusion there was a trace of histamine in the second half-hour sample, about 1 : 40 millions; the other six samples were free from detectable histamine. The drainage fluid contained 1 : 12 millions histamine. The lung now weighed 47 gm. having increased 62 p.c. by oedema. The right lung contained 25γ and the left lung 28γ histamine per gm. The S.R.S. was maximal during the first half-hour of administration of ethyl chloride and gradually diminished in successive samples, being absent in the last. There was much S.R.S. in the drainage fluid.

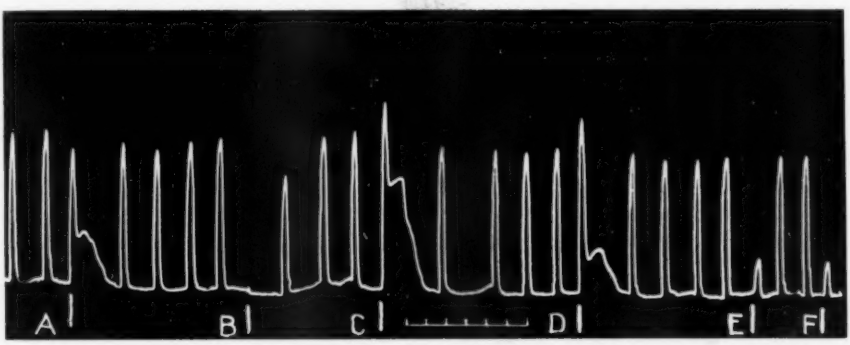

Fig. 7. Responses of the isolated jejunum of a guinea-pig to perfusate and drainage fluid from a dog's lung ventilated with 44 p.c. ethyl chloride for half-an-hour. The unlettered responses are to 0·1γ histamine. Time in minutes. Details in text.

That smaller concentrations of ethyl chloride were without effect is illustrated by the following experiment, in which a lung weighing 55 gm. was ventilated with 22 p.c. ethyl chloride for half-an-hour. The samples during four hours' perfusion and the drainage fluid contained neither histamine nor S.R.S. in detectable amounts. There was no oedema of the lung. The histamine content of the right lung was 22γ, and of the left after perfusion 23γ per gm.

Ethylene.

Ethylene 80 p.c. administered with oxygen for one or two hours caused very slight injury to the perfused lung, evidenced by the liberation of small amounts of histamine during three hours' perfusion after the administration. Administered for two hours in 95 p.c. strength with oxygen a trace of histamine appeared towards the end of the perfusion, and given pure without oxygen for two hours there was in one experiment a small continued output of histamine. In none of these experiments was there a detectable increase in the protein in the perfusate, and in none was S.R.S. set free.

The largest output of histamine with 80 p.c. ethylene was observed in an experiment on a lung weighing 53 gm. The perfusate before and during the administration (one hour) and during the first half-hour afterwards was free from detectable histamine. The succeeding half-hour samples contained 1 : 40 millions, 1 : 30 millions and 1 : 40 millions histamine. The drainage fluid contained 1 : 20 millions—the total output of histamine being 3γ. The lung now weighed 76 gm., an increase in weight from oedema of 43 p.c. The right lung contained 80γ per gm. and the left lung 67γ per gm. In two other experiments the perfused lungs weighed 36 and 38 gm. and the right lungs contained 13γ and 40γ histamine per gm. respectively. Ethylene 80 p.c. administered for two hours caused no detectable output of histamine—the drainage fluid after 2½ hours' further perfusion containing 1 : 50 millions and 1 : 30 millions histamine.

When ethylene 95 p.c. was administered with oxygen for two hours to a lung weighing 55 gm. traces of histamine were found in the perfusate during the second hour of administration and for 1½ hours afterwards, the last sample containing 1 : 30 millions histamine (1γ). The drainage fluid contained 1 : 12 millions histamine (1γ) and the total output was 3γ. The lung now weighed 60 gm., an increase from oedema of 9 p.c. The right lung contained 120γ histamine and the left lung 90γ per gm.

Ethylene without oxygen gave in one experiment a rather larger output of histamine. During the second hour of administration the histamine in the perfusate was 1 : 35 millions (2γ), and during the next two half-hour periods there were 1 : 22 millions (2γ) and 1 : 14 millions (2·5γ) of histamine in the perfusate. The drainage fluid contained 1 : 7 millions (1·5γ). The total output of histamine in three hours' perfusion was 6γ. The right lung contained 70γ and the left lung after perfusion 67γ per gm.

Nitrous Oxide.

This anaesthetic administered for two hours in 80 p.c. strength with oxygen caused no evident injury to the perfused lung, the perfusate being free from histamine and S.R.S. and the drainage fluid containing only small amounts of histamine. Administered in 90 p.c. or 95 p.c. strength with oxygen or pure, small amounts of histamine appeared in the perfusate after the administration. No S.R.S. was set free in any of the experiments.

A lung weighing 46 gm. was ventilated for two hours with a mixture of 80 p.c. nitrous oxide and 20 p.c. oxygen. At no time during 4½ hours' perfusion was there any detectable histamine or slow reacting substance in the perfusate. The drainage fluid contained 1 : 50 millions histamine. The lung now weighed 54 gm., its weight having increased 17 p.c. by oedema. The right lung contained 15γ and the left after perfusion 20γ histamine per gm. In another similar experiment in which the lung contained 48γ histamine per gm., the perfusate was free from histamine,

but the drainage fluid contained 1 : 20 millions histamine. There was no oedema of the lung.

The effects of higher concentrations of nitrous oxide are illustrated by the following experiments. A lung weighing 48 gm. was ventilated with a mixture of 90 p.c. nitrous oxide and 10 p.c. oxygen for two hours. The perfusate before and during administration was free from histamine. The succeeding half-hour samples contained 1 : 50 millions (0·5γ), 1 : 35 millions (0·8γ), 1 : 30 millions (0·8γ), 1 : 30 millions (0·7γ), 1 : 28 millions (1·0γ) and 1 : 30 millions (1γ). The drainage fluid contained 1 : 10 millions histamine (1γ) and the total output was 6γ. No slow reacting substance was detected in the perfusate or in the drainage fluid. The lung now weighed 59 gm., having increased 23 p.c. by oedema. The right lung contained 50γ and the left after perfusion 55γ histamine per gm.

A lung weighing 29 gm. was ventilated with nitrous oxide 95 p.c. and oxygen 5 p.c. for two hours. During an hour's perfusion before and during the administration no histamine was present in the perfusate. During the period half to one hour after 1 : 40 millions histamine was found. At the end of the experiment the lung weighed 36 gm., having increased 24 p.c. by oedema. The right lung contained 57γ and the left after perfusion 55γ histamine per gm. There was no output of slow reacting substance.

A lung weighing 26 gm. was ventilated with pure nitrous oxide for two hours. No histamine appeared in the perfusate during the administration of the gas, but in the two succeeding half-hour samples 1 : 35 millions (1·3γ) and 1 : 30 millions (1·2γ) were found and the drainage fluid contained 1 : 6 millions. The total output was 3γ. The lung now weighed 30 gm., an increase by oedema of 15 p.c. The right lung and the left lung after perfusion contained only 17γ histamine per gm.

DISCUSSION.

The agents which are capable of causing injury to tissue cells with the liberation of histamine from isolated organs appear to fall into two groups according to whether S.R.S. does or does not appear in the perfusate. To the former class belong the snake venoms, staphylococcal toxin and the anaphylactic antigen in the sensitized animal and to the latter, radiant energy, photodynamic action and mercuric chloride.

Examination of perfusates from the isolated dog's lungs ventilated with volatile anaesthetics (chloroform, ether and ethyl chloride) shows that with each, histamine and S.R.S. are liberated when the concentration of vapour rises above certain values. These anaesthetics must therefore belong to the first group, and it is to be presumed that they possess something in common with the other noxious agents in this group. Snake venoms act powerfully upon the lipins of the cell to form lysocithin and probably alter the permeability of the lipin layers in the cell. The ready solubility of these anaesthetics in the lipins has been held by Meyer (1899)

and Overton (1901) to be the basis of their anaesthetic action. When snake venoms in concentrations too small to cause liberation of histamine act upon isolated organs S.R.S. may still appear in the perfusate (Feldberg and Kellaway, 1938). Ether and ethyl chloride behave similarly, causing the liberation of S.R.S. with concentrations too small to liberate histamine (Fig. 8), whereas with chloroform histamine is liberated by concentrations lower than those needed to set free S.R.S.

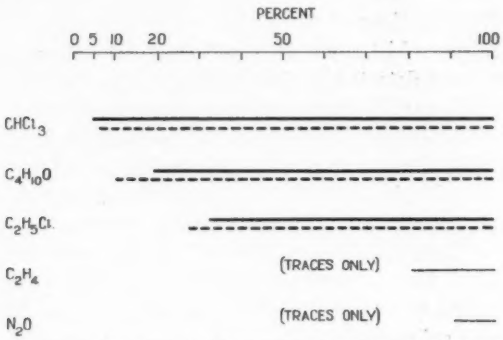

Fig. 8. Diagram illustrating output of histamine (continuous line) and of S.R.S. (dotted line) in response to various percentages of anaesthetics.

Though the injury caused by the gaseous anaesthetics (ethylene and nitrous oxide) is very slight, they must be placed in the second group since no S.R.S. is set free by their action.

The artificial conditions obtaining when the lung is perfused with Tyrode solution for several hours make the onset of oedema almost inevitable, and the drainage fluid obtained after the lung has been cut down usually contains some histamine. In estimating the degree of injury to the lung we may therefore disregard the oedema observed in these experiments, and even the presence of histamine in the drainage fluid unless it reaches a concentration of 1 : 15 millions or stronger.

Ether as employed in the open method according to Hewitt and Symes (1912) may reach percentages ranging from 5–15 p.c. According to Waller (1911) the ether percentage inhaled from an open mask ranges from 8–16 p.c. These percentages reach the limit at which in our experiments a minor degree of tissue injury can be demonstrated by the liberation of S.R.S. To produce severe degrees of injury higher concentrations of ether are required. It is possible that some injury may be caused in the lung during induction if percentages as high as 15 are required for this purpose. The administration of ether to a lung already damaged by disease presents a different problem. It is well known that ether anaesthesia may be harmful in persons with pulmonary disease, and this may be related to the minor degree of tissue injury revealed by liberation of S.R.S. from the isolated

dog's lung ventilated with ether (10–15 p.c.). Ether in these concentrations in the intact cat probably causes some changes in the permeability of endothelial tissues, for Dale (1919) showed that histamine shock is readily produced in these animals under ether anaesthesia, whereas under anaesthesia with nitrous oxide and oxygen they are almost as resistant to histamine poisoning as when they are unanaesthetized. This may possibly be dependent upon the local action of S.R.S. liberated in the tissues under ether anaesthesia, no S.R.S. being set free by nitrous oxide, or upon the trivial degree of injury caused by this latter anaesthetic.

Chloroform anaesthesia is usually induced with 2–2·5 or even 3 p.c. (Alcock, 1909), and the percentages used to maintain it must be smaller. Within these limits of concentration there was no appreciable injury to the perfused lung in our experiments. The injurious effects of chloroform on the lung as shown by the liberation of histamine and S.R.S. only appear with concentrations (4–5 p.c.), which are lethal when administered to animals or man. Our experiments, though they fail to show any immediate injury with concentrations ranging from 2·2–3·8 p.c., do not exclude the possibility of delayed effects.

We were unable to detect any significant injury to the perfused lung ventilated for half-an-hour with ethyl chloride 22 p.c. Since Hewitt (1904) has shown that concentrations of ethyl chloride from 9–12 p.c. are adequate to produce anaesthesia, and since such anaesthesia is usually only of short duration, a directly injurious effect on the tissues of the lung is unlikely.

The administration of ethylene 80 p.c. with oxygen caused only trivial injury to the perfused lung, and even in higher concentrations the degree of injury as judged by our perfusion experiments was slight. Nitrous oxide 80 p.c. administered with oxygen is without any injurious effect upon the isolated perfused lung of the dog. When administered in higher concentrations a small output of histamine is observed in the perfusate. Since ventilation of the lung with air, or even with nitrogen (99·8 p.c.) causes no output of histamine, the results with high concentrations of nitrous oxide and ethylene must be regarded as significant.

CONCLUSIONS.

Volatile anaesthetics cause severe injury to the tissues of the perfused lung of the dog with liberation of histamine and S.R.S., only when administered in high concentrations. These concentrations were: For ether 19–44 p.c., for chloroform 4·4–8 p.c. and for ethyl chloride about 40 p.c. Moderate concentrations of ether 10–16 p.c. caused the liberation of S.R.S. but not of histamine; chloroform in concentrations of 2·2–3·8 p.c. and ethyl chloride in concentrations up to 20 p.c. were without effect.

The gaseous anaesthetics tested, ethylene and nitrous oxide, even when administered pure without oxygen, cause no liberation of S.R.S. and only trivial outputs of histamine from the perfused lung of the dog.

ACKNOWLEDGMENTS.

By the courtesy of Mr. Russell Grimwade we received some free supplies of nitrous oxide and ethylene from Australian Oxygen and Industrial Gases, which firm also kindly lent us an anaesthetic machine for these experiments.

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PHYSIOLOGICAL ONTOGENY IN THE TOBACCO PLANT[1]

2. THE DRIFTS IN WATER CONTENT OF THE LEAVES IN RELATION TO PHOSPHORUS SUPPLY AND TOPPING.

by

RUTH WATSON

(From the Waite Agricultural Research Institute, University of Adelaide).

(Submitted for publication 31st July, 1939.)

INTRODUCTION.

In the first paper of this series (Petrie, Watson and Ward, 1939) a study was made of the progressive changes in the dry weights and leaf areas of tobacco plants as affected by phosphorus supply and topping. This second paper is devoted to the consideration of the drifting water content of the leaves in the same experiment. Water contents were calculated from the differences between fresh and dry weights of each of the six replicates of leaf material, mean values being presented in the graphs. The treatments I–IV were progressively increasing supplies of phosphorus; all other experimental details were given in the previous paper.

ANALYSES OF VARIANCE.

Analyses of variance were carried out on the percentage water data for harvests 5 and 6 in the case of the first leaf group, and for harvests 5–7 in the cases of the second and third groups. The values for harvests 4a and 4b were omitted since they differed considerably and could not be allotted to the same harvest class.

The results of the analyses show that, in each group, the average percentage water content for treatments II, III and IV combined is significantly greater than that for treatment I. Also the mean percentage water content for the untopped plants is significantly greater than that for the topped plants in all three groups. The significances relate to the 5 p.c. point, as was also the case for the analyses in the previous paper.

ABSOLUTE WATER CONTENTS.

Total Leaves (Figs. 1 and 2).

At harvest 2 the value for treatment IV shows the characteristic initial depression as in the dry-weight and leaf-area data; thereafter the values are lowest

[1] The work described in this paper was carried out as part of a general investigation on quality in tobacco undertaken by the Commonwealth Council for Scientific and Industrial Research.

with treatment I. In the untopped plants the curve for treatment I has an earlier maximum than those for the remaining treatments,[2] but, on the other hand, falls less rapidly subsequent to the maximum. The curves show a considerable similarity to those of the leaf area (Petrie, Watson and Ward, Fig. 9), and have maxima at the same times. The dry-weight curves for the higher treatments, on the con-

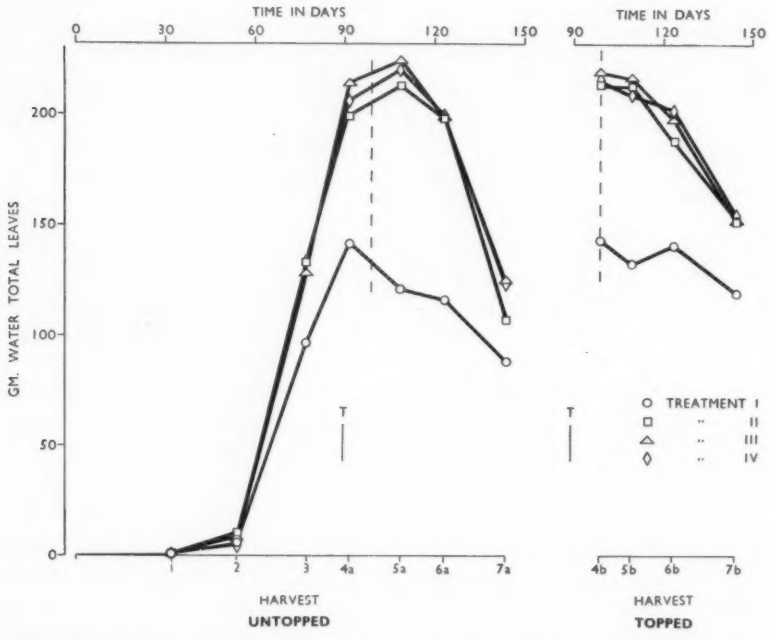

Fig. 1. Curves of absolute amounts of water in the total leaves (gm. per plant). The vertical lines marked T in this and succeeding graphs indicate the point in time at which topping was performed; for comparative purposes this point in time has been marked in the graphs for the untopped plants as well as those for the topped plants. The dotted lines indicate corresponding points in time on the curves for the topped and untopped plants.

trary, continue to rise until the end of the experiment, and the fall with treatment I could not be shown to be significant (Petrie, Watson and Ward, Fig. 3).

Topping has reduced the rate of fall in the amount of water in the leaves with all treatments, the effect being greater and appearing earlier with treatment I. Topping has not, however, affected the maximum water values, although it caused a great increase in the accumulation of dry matter (Petrie, Watson and Ward, Fig. 4).

[2] This is not apparent in the individual leaf groups, but may be accounted for by the relatively large decline between harvests 4a and 5a in the curve for the first group (Fig. 3).

The Leaf Groups (Figs. 3 and 4).

The curve for treatment I is lowest in all groups, as was the case with the dry weights. In this connection it must be recalled that the number of leaves varies with treatment in the third and fourth groups (cf. Petrie, Watson and Ward, p. 103).

Topping has had the smallest effect on the first group of leaves, has considerably reduced the senescent fall in the second, and has caused a large increase up to the final harvest in the third group; in the second and third groups the effect

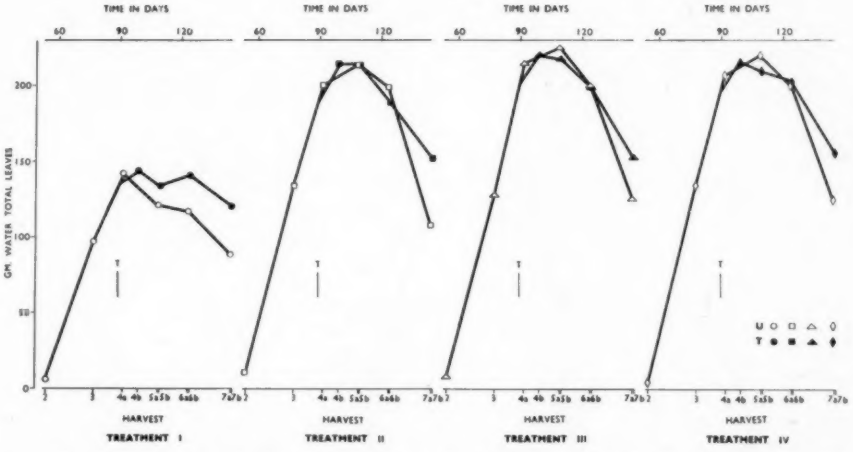

Fig. 2. Curves of absolute amounts of water (gm. per plant) in the total leaves, the curves for the topped and untopped plants superimposed. In this and succeeding graphs **U** ≡ untopped and **T** ≡ topped.

appears earlier with treatment I. In these respects the water curves tend to resemble those of the leaf area (cf. Petrie, Watson and Ward, Figs. 10 and 11). In each group the increases in dry weight produced by topping are of a relatively greater magnitude than those in area or absolute water, especially in the third group.

PERCENTAGE WATER CONTENTS.

Total Leaves (Figs. 5 and 6).

There is a continuous decline with time in the percentage water content from harvest 1. The values with treatment I are in general the lowest, and at harvest 2 there is a temporary depression with treatment IV.

Topping has had the general effect of lowering the percentage water content.

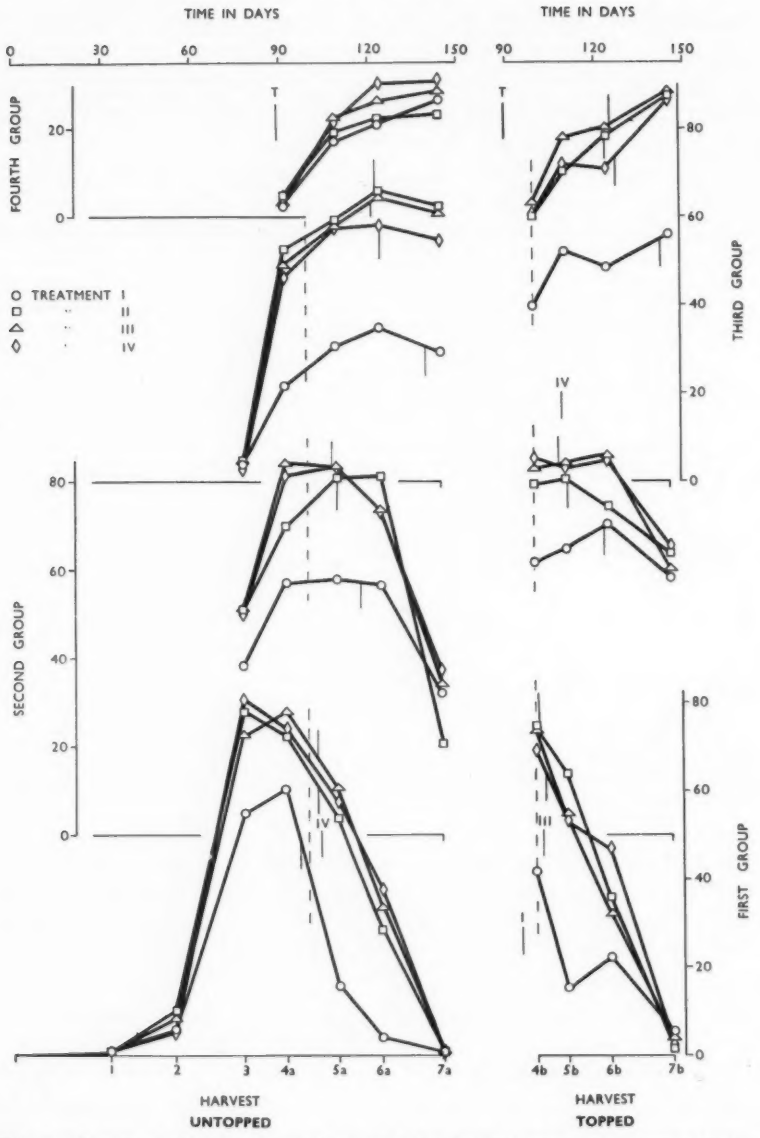

Fig. 3. Curves of absolute amounts of water in the leaf groups (gm. per plant). The unbroken vertical lines (where necessary marked with appropriate treatment number) in this and succeeding graphs indicate the average time of attainment of ripeness for the group of leaves concerned.

The Leaf Groups (Figs. 7 and 8).

In general the curves show a decline with time; the low values for all treatments at harvest 2 in the first group may be partly due to the temporary set-back produced by transplanting. In each group the curves with treatment I are the lowest throughout the greater part of the period of observation; in the late stages, however, an intersection is suggested in all groups of the topped plants and in the second group of the untopped plants, but is not attained in the third and fourth groups of the untopped plants within the time of the experiment.

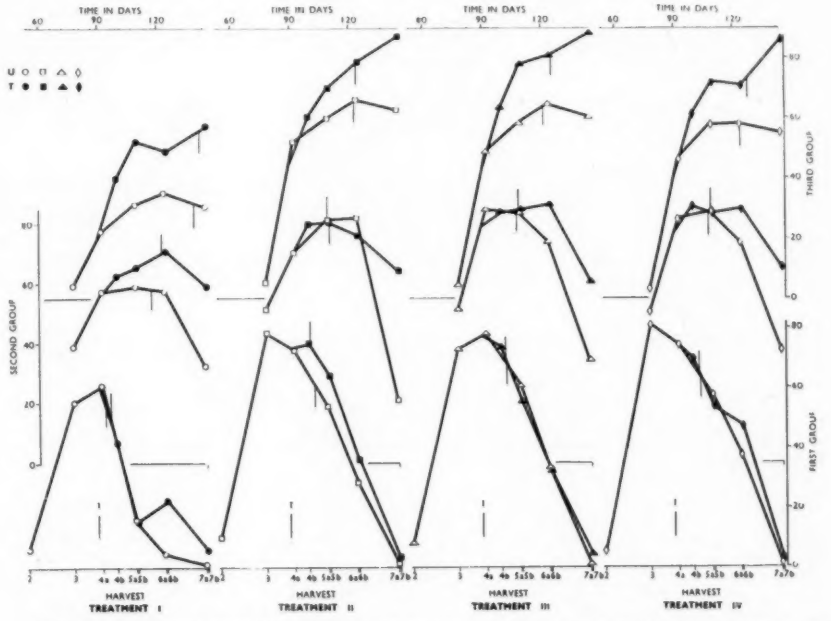

Fig. 4. Curves of absolute amounts of water in the leaf groups (gm. per plant), the curves for the topped and untopped plants superimposed.

Combining the treatment data for the harvests included in the analyses shows that topping has significantly reduced the percentage water content in each of the three groups; the graph, however, shows no reduction in the case of the first group with treatment I.

DISCUSSION.

The Effect of Treatment.

In the work of Williams (1936, 1938), to which reference was made in the previous paper, it was found that senescent loss of water commenced earlier in

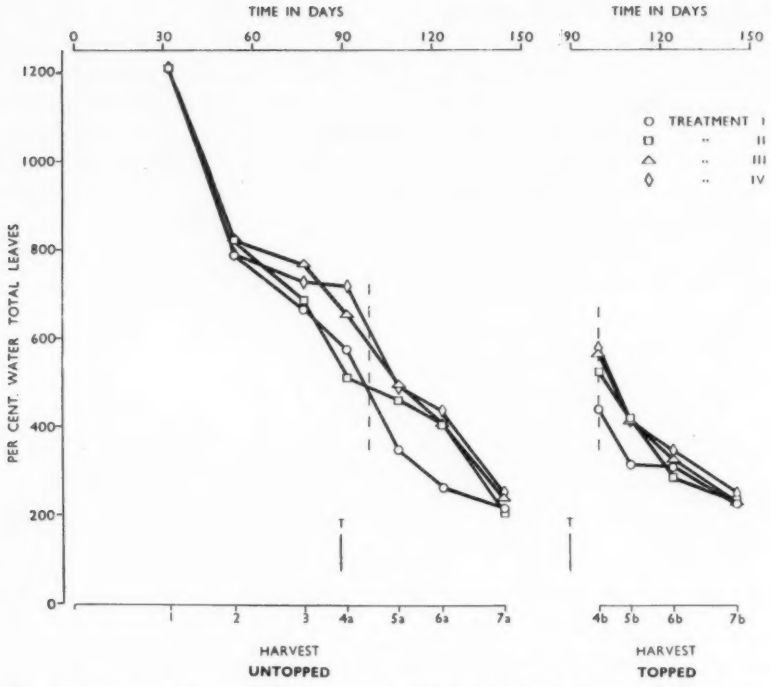

Fig. 5. Curves of percentage amounts of water in the total leaves (gm. per 100 gm. dry matter).

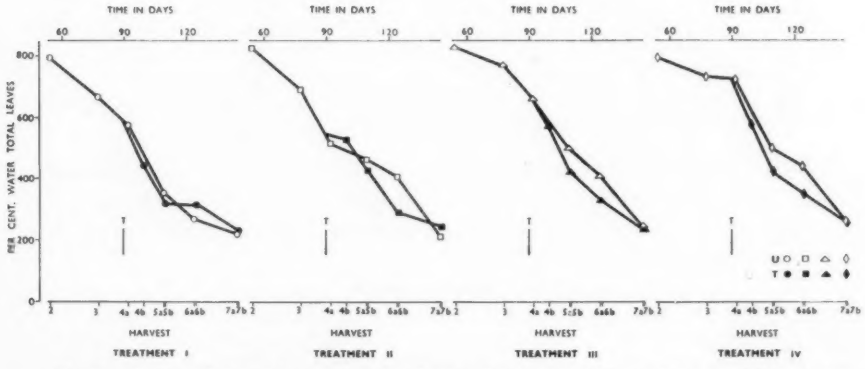

Fig. 6. Curves of percentage amounts of water in the total leaves (gm. per 100 gm. dry matter), the curves for the topped and untopped plants superimposed.

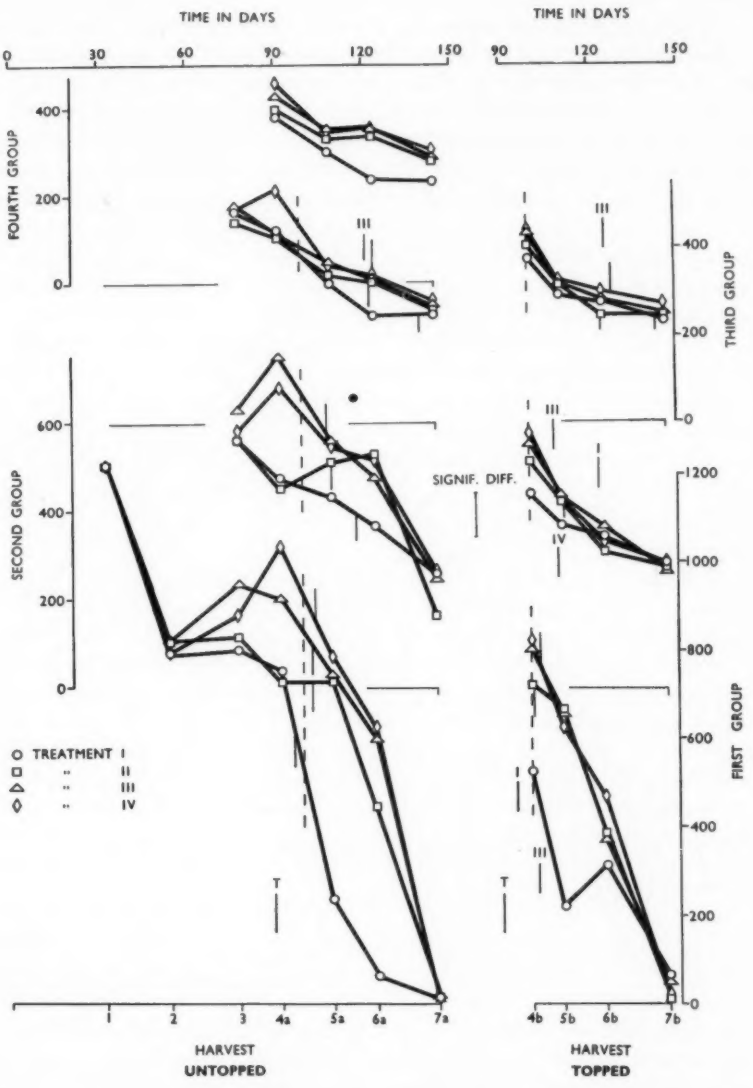

Fig. 7. Curves of percentage amounts of water in the leaf groups (gm. per 100 gm. dry matter). The significant difference for the second group represents 2·828 × the standard error of the mean of 6 replicates, and is applicable to the comparison of individual points lying between harvests 5–7 inclusive.

plants receiving high phosphorus supply, a fact attributed to their relative nitrogen deficiency. The percentage water content was at first increased with increasing phosphorus supply; this was evidently a specific effect of phosphorus treatment, and was correlated with an increase in protein content. The earlier senescence, however, led to a reversal of the picture, the high phosphorus plants ultimately having the lower water content. Williams's results show also that increasing phosphorus supply ultimately produced a relatively greater increase in dry weight than in absolute water content. This suggests that with high phosphorus treat-

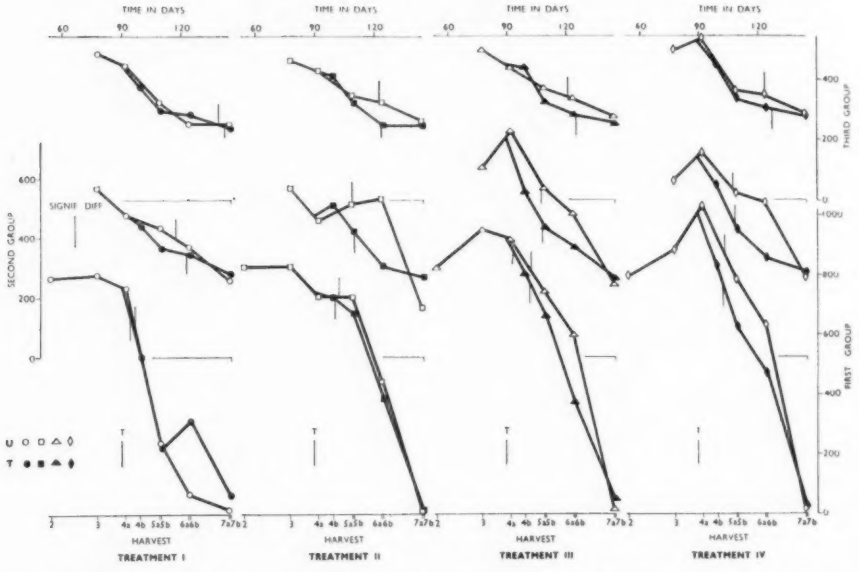

Fig. 8. Curves of percentage amounts of water (gm. per 100 gm. dry matter) in the leaf groups, the curves for the topped and untopped plants superimposed. The significant difference is as described in the legend to Fig. 7.

ment the later increments of dry matter were relatively less able to be utilized in growth of cells, and hence may have accumulated to a greater extent as carbohydrate reserves. Had a larger supply of nitrogen been available in Williams's experiments, nitrogen deficiency of the high phosphorus plants might not have become acute enough to cause hastening of senescence or intersection of the curves. Sommer (1936) showed that plants grown with a plentiful nitrogen supply matured earlier when the phosphorus supply was low; and Richards and Templeman (1936) found that in individual barley leaves at and after maturity the water content was reduced by phosphorus deficiency.

In the present work the first group of leaves exhibits with low phosphorus supply an earlier decline in absolute water content and a persistently lower percentage water content. Earlier senescence with low phosphorus supply was also suggested by the data for time of ripening, as was pointed out in the previous paper. Probably in this group of leaves an adequate amount of nitrogen was available, and the treatment effects are more direct consequences of the differences in phosphorus supply. In the second group of leaves, and generally in the leaves of the topped plants, decline in absolute water content tends to be earlier with high phosphorus supply; and in all but the first group the percentage water-content curves either converge or intersect in the manner found by Williams. These may be cases where nitrogen deficiency was beginning to have an effect in the plants receiving the higher phosphorus treatments. The experiment was terminated before such effects had become pronounced in the third and fourth groups.

The decreased percentage water content with phosphorus deficiency may be related to two factors. It may be partly determined in a direct manner by decreased percentage protein content, and it may also be the result of accumulation of carbohydrates which were unable to be used in normal growth on account of limitation of phosphorus supply.

The Effect of Topping.

In the previous paper it was pointed out that assimilates, which in the untopped plants went to produce the inflorescence, in the topped plants remained partly in the leaves; consequently the final dry weight of the leaves was greater in the topped plants, the more so the higher the position on the stem. This topping effect was relatively greater on the dry weight than on the area (cf. Petrie, Watson and Ward, Fig. 13); the increased dry weight in the topped plants must therefore have been due partly to accumulation of reserve materials within the cells or to a relatively greater formation of mechanical tissue. This accumulation would cause the observed reduction in percentage water content. Probably no significance can be attached to the apparently exceptional behaviour with treatment I in the first group.

The Position on the Stem.

The mean values of the water content with the four treatments at the time of attainment of maximum leaf area are as follows:

first group	821 p.c.
second „	562 „
third „	319 „
fourth „	280 „

There is a decline in the water content of the successive leaf groups from the base to the apex of the stem, and the detailed data show that this holds with each treatment. This was observed for individual leaves by Krasnoselsky-Maximov (1917),

Richards and Templeman (1936) and Portsmouth (1937); it is related to structural differences in the successive leaves which are described in some detail by Maximov (1929).

The Relation of Water Content to Leaf Area.

The correlation between leaf area and absolute water content is apparent from comparison of Fig. 10 of the previous paper with the present Fig. 3. We may perhaps regard both attributes as measures of the expansion of the cells and as attaining their maxima at the time of cessation of cell enlargement.

The ratio of absolute water content to leaf area is an approximate measure of the thickness of the leaves, and it is found that topping increases this quantity by about 17 p.c. in the third group of leaves; this agrees qualitatively with the conclusions of Avery (1934) based on direct measurements of leaf thickness.

Richards (1932), from an analysis of data on individual mature barley leaves, concluded that manurial effects on the dry weight per unit area were mainly due to variations in the degree to which the leaves were inflated with water. On the other hand, Richards found that differences in the ratio of dry weight per unit area in the successive leaves could not be accounted for in the same way, which indicated that these leaves differed structurally. The data of the present experiment are insufficiently extensive to test whether these conclusions apply here also.

SUMMARY.

This paper deals with the drifting water content of the leaves of the tobacco plant as affected by phosphorus supply and topping.

Absolute water content was increased with high phosphorus supply to a relatively greater extent than the dry weight; that is, treatment increased the percentage water content. Senescent fall in absolute water content, however, tended to be greater with higher phosphorus treatment, so that there was a tendency for a final convergence or intersection of the percentage water content curve for low phosphorus treatment with those for the higher phosphorus treatments. This phenomenon is attributed to relative nitrogen deficiency in the plants receiving high phosphorus supplies. An exception occurred in the leaves at the base of the stem; here relative nitrogen deficiency presumably did not occur, and senescence was more rapid with low phosphorus supply.

Topping increased the absolute water content, but to a relatively less extent than the dry weight, so that in general it decreased the percentage water content. It is concluded that assimilates in the upper leaves of the topped plants, which normally would have been translocated to the inflorescence, were partly accumulated as reserve materials or as mechanical tissue.

In general there was a progressive decline in percentage water content of the leaves at their maximum area from the base to the apex of the stem.

There was a marked correlation between leaf area and absolute water content.

ACKNOWLEDGMENTS.

The writer desires to acknowledge her indebtedness to Dr. A. H. K. Petrie and to Mr. R. F. Williams for their critical advice on the interpretation of the results and in the preparation of the manuscript.

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POLIOMYELITIS

1. INTRAOCULAR INOCULATION AS A STANDARD METHOD FOR THE DEMONSTRATION OF NEUTRALIZING ANTIBODIES

by

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(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 6th May, 1939.)

In all previous work on the neutralizing power of human serum for poliomyelitis virus the experimental method has been to inoculate serum-virus mixtures intracerebrally in young rhesus monkeys. Owing to the expense of these animals, very small numbers (often one, and rarely more than two) have been used for each test, and most workers have been satisfied to use a single mixture of undiluted serum with a standard virus preparation. Such methods would never be contemplated by workers accustomed to research on viruses which can be titrated with freely available test objects like mice and developing eggs, were any more accurate method available. If monkeys are the only susceptible animals, greater accuracy can be obtained either by the use of larger numbers of animals or by using a more satisfactory method of inoculation. Schaeffer and Muckenfuss (1938) have recently pointed out the variability of results obtained in endeavouring to titrate sera accurately by the intracerebral method, and have shown that inocula of easily recognizable substances such as India ink may spread to very different parts of the central nervous system. Results confirming this variability will be presented in the present communication.

In considering whether any alternative method of inoculation might overcome these difficulties, we are limited to methods bringing the virus into direct contact with nerve cells, since all other methods are notoriously irregular in their results. The only route which seemed to us at all promising was the intraocular one. By depositing the serum-virus mixture against the retina, one should be able to ensure (1) that it was in contact with susceptible cells, and (2) that the inoculum would remain under uniform conditions as regards dilution with body fluids and diffusion from the site of inoculation. It was this second factor that offered a hope that results by the intraocular method would be more consistent than those by intracerebral inoculation. For a discussion of the part played by such factors in determining the results of serum neutralization tests the monograph by Burnet, Keogh and Lush (1937) may be consulted. This hope has been to a considerable extent fulfilled, and we have adopted the method as our standard method of antibody titration.

[1] Work carried out under grant for poliomyelitis research from the National Health and Medical Research Council.

PRELIMINARY EXPERIMENT.

The first small experiment with the method was made in January, 1938, and as the results were typical of those obtained in later experiments they will be described in some detail. Six monkeys (*M. rhesus*) were used, two being inoculated with a stock virus suspension of the Rockefeller Institute strain M.V. diluted 1 : 2 with saline, two with virus plus an equal volume of pooled serum from monkeys which, after recovery from infection with one or other strain of local virus, had been re-inoculated with M.V. and had survived. The other two received stock local virus "Mar". The following notes indicate the results:

Monkey 1. M.V. 1: 2. Temperature 105·1°F. on the fourth day; showed definite bilateral ptosis, the eyelids drooping to about half way across the eyeball. The animal was killed for histological study.

Monkey 2. M.V. 1: 2. Temperature 105·0°F. on the seventh day; bilateral ptosis well marked; excitable with moderate tremor, but no paralysis of limbs, eyes react to light, and no evidence of weakness of extrinsic muscles of eyes. On the following (eighth) day head was turned to the right and there was some weakness in all limbs, but particularly in right arm. Killed on this day.

Monkeys 3 and 4. M.V. plus pooled immune monkey serum. Monkey 3 showed on the ninth day a temperature of 105·1°F., but no symptoms and remained well, the other showed neither symptoms nor temperature.

Monkey 5. "Mar" local virus. Temperature 104·2°F. on the seventh day and 105·1°F. on the eighth day, no symptoms till the ninth day, when a slight tremor appeared. On the following day weakness in the lower limbs was apparent, and a fairly severe paralysis of both legs persisted. The arms remained unaffected and the animal was allowed to survive. There was no sign of cranial nerve involvement at any time.

Monkey 6. "Mar". No temperature rise to the twelfth day. Temperatures were not then taken until the sixteenth day, when monkey showed ruffled fur and right-sided facial paralysis, with temperature 106·7°F. On the following day tremor was very gross and movements of the arms were ataxic. Next day (eighteenth) nearly complete paralysis of left arm and leg with complete right facial paralysis: animal killed.

This experiment appeared to indicate first that the retina was highly susceptible to infection, and that symptoms appeared almost as rapidly as after intracerebral inoculation; second, that neutralization tests could be made by the method; and third, that with the strain M.V. at least infection produced the interesting syndrome of early bilateral ptosis. Further experiments were therefore planned to see whether the method could be adopted as a standard one, and also to elucidate the nature of the lesions producing the early ptosis. In this paper we are concerned only with the experiments using the M.V. strain. Subsequent intraocular inoculations of two monkeys with "Mar" failed to produce infection, and work with this strain was largely dropped until the high susceptibility of *Macacus cynomolgus* to the virus was discovered. This species is highly susceptible to intraocular inoculation of "Mar", but it will be more convenient to report all work with cynomolgus monkeys in a separate paper.

TECHNIQUE.

The monkey is anaesthetized with ether and placed on its right side. The left eye is swabbed clean with saline and the bulbar conjunctiva grasped with a pair of small conjunctival forceps, just outside the corneosclerotic junction on the inner side of the eyeball. A tuberculin syringe fitted with a freshly sharpened needle, gauge 25, and containing 0·10 c.c. of inoculum, is used. The needle is pushed through the cornea and iris with a rotary motion, and then directed gently toward the retina on the medial side of the eyeball. When the resistance of the sclerotic is felt, the inoculum is discharged and the needle is withdrawn. There is usually some leakage of aqueous humour, but tests with India ink show that the inoculum is deposited only in the posterior chamber. On recovery from the anaesthetic the animals give no evidence of being in pain.

Symptoms Following Infection by the Intraocular Route.

The appearance of bilateral ptosis as the initial symptom has not been invariable, and there have been rather striking differences in the proportion of monkeys showing the sign at different stages of our work. It was comparatively rare amongst monkeys inoculated with M.V. in May and June, 1938, e.g. of the eight animals in Table 1 paralysed after intraocular inoculation, only four showed ptosis. Inoculations made in November and December, on the other hand, resulted in ptosis in sixteen out of seventeen infections, which eventually produced definite paralysis. The one exception was also the only monkey of the series inoculated with M.V., which survived after being definitely paralysed in the legs. The strain of virus, species and source of monkeys and method of inoculation and handling were the same throughout, so that the difference must be ascribed to some minor variable.

The ptosis might appear on the same day as the first significant rise of temperature, but in several instances was one or two days later. The usual early signs of infection, tremor and excitability, tended to be more closely related to the first rise of temperature. The degree to which the eyelids droop varies from monkey to monkey, but the ptosis has always been bilateral and approximately symmetrical. Since very little of the upper eyelid is visible in a normal rhesus monkey, even a moderate degree of ptosis gives a characteristic and easily recognized expression to the animal. Apart from possible weakness of upward movement, we have observed no associated weakness of the extrinsic eye muscles, nor any pupillary changes in the uninoculated eye. Apart from the ptosis, the clinical picture is very similar to that following intracerebral inoculation; paralysis usually appears first in the lower limbs and advances in 90 p.c. of animals to complete prostration and death. The time elapsing from the appearance of ptosis to complete prostration varied, but was usually three or four days. Amongst sixteen animals the following periods between ptosis and prostration were noted: 2, 3, 3, 3, 3, 3, 4, 4, 4, 4, 4, 5, 5, 5, 6, 28 days. The last animal was definitely paralysed three days after ptosis, and the final prostration 25 days later was probably one of the rare examples of relapse.

Comparative Titration of Poliomyelitis Virus and Antibody by Intracerebral and Intraocular Methods.

For each main series of experiments we have prepared a considerable stock of virus in the form of a coarsely-ground emulsion of several spinal cords in lightly buffered 50 p.c. glycerol Ringer solution pH 7·4. This suspension (one part tissue to two parts glycerol-saline) was distributed to tubes in 1 c.c. amounts, and these were stored at a temperature of — 2° to 0° C. For each experiment 1 c.c. of the suspension was mixed with quartz powder and finely ground with pestle and mortar and 9 c.c. of nutrient broth added. The emulsion was centrifuged for five minutes in an angle centrifuge at 3,000 r.p.m. and the supernatant fluid used as stock virus. The dilutions shown are in terms of this stock material.

Table 1 shows a comparative titration of the same stock of M.V. by the two methods of inoculation. The dilutions were prepared in ice-cold nutrient broth and kept cold until just before inoculation. The intracerebral injections were of 0·25 c.c., the intraocular ones of 0·10 c.c. From these results, and allowing for the greater volume of the intracerebral inoculum, the intracerebral method is about four times as sensitive as the intraocular. Another sample of the same stock virus was tested intraocularly three months later at the conclusion of the experiments reported in this paper, and showed no apparent loss of activity, both monkeys inoculated with 1 : 100 virus showing typical symptoms at nine and ten days.

TABLE 1.

Comparative Titration of M.V. Virus by Two Methods.

	Undiluted.	10^{-1}	10^{-2}	10^{-3}
Intracerebral	—	8, 8, 9	8, 10, 11	12, 12, S
Intraocular	8, 10, 8	8, 9, 10	9, 12, S	S, S, S

Day of definite paralysis shown.
S = survival without symptoms.

A detailed titration of a pooled human convalescent serum was carried out by both methods with the results shown in Table 2. This serum, C.S.L. Batch 81, was made available by the courtesy of Dr. John Dale and Dr. F. G. Morgan. It was obtained from six individuals infected during the 1937–8 epidemic, and contained no antiseptic. Tests were made by mixing suitable dilutions of serum with stock virus prepared as described above. The mixtures were kept in the refrigerator or in an ice bath until the inoculation was made, the time elapsing between mixing and injection being from 1½ to 3 hours. There seems to be no theoretical or practical justification for the common practice of incubating for a short period at 37° C. followed by a sojourn in the refrigerator overnight. Our use of refrigerator temperature and a period of 1–3 hours' contact is based on experience obtained during immunological studies with several viruses titrable on the chorioallantois (Burnet, Keogh and Lush, 1937). It was found that these conditions are a suitable com-

promise to obtain, with a wide variety of serum and virus concentrations, results which are not vitiated by spontaneous destruction of virus or by insufficient time being allowed to reach an equilibrium level.

Table 2 shows the results obtained using six monkeys in two experiments for each of the dilutions near the end point.

TABLE 2.

Comparative Antibody Titrations of Two Sera Against M.V. by Intracerebral and Intraocular Methods.

	Batch 81.		Batch 82.	
	Intracerebral.	Intraocular.	Intracerebral.	Intraocular.
Virus + Undiluted serum	—	—	S, ±	±, ±, S
+ Serum 1: 5	S, S, S, 12, 4	S, S,	7, 8	4, 9, ±
1: 25	S, S, 8, 8, 9	S, S, S, S, S, S	8, 8	8, 9, 9
1: 125	6, 11	S, S, S, S, 14, ×	8, 9	—

± = temperature rise not followed by paralysis.
× = nonspecific death.

It will be seen that the results with the intracerebral test are most irregular and indicate a much lower degree of antibody activity than is shown by the intraocular method. Serum diluted 1 : 125 plus undiluted virus infected only one monkey in five, and all stronger concentrations of serum gave protection. In the same table is shown as contrast the results obtained with another batch of convalescent serum, C.S.L. No. 82, taken from two individuals only. This shows partial protection only with undiluted serum, so that the antibody content can be taken as approximately 1 : 100 of that in serum 81.

This comparison was of considerable interest, first as establishing the practicability of the intraocular method, and second as showing that "convalescent serum" which had been taken for therapeutic use might, if taken from only a few individuals, contain considerably less antibody than is present in normal pooled adult serum. Further experience of the method in titrations of individual sera from patients with poliomyelitis and contacts, which will be reported in a subsequent paper, has confirmed its suitability as a standard method for the purpose, provided always that the well adapted M.V. strain is used.

The Histology of the Lesions Following Intraocular Inoculation.

The lesions produced in the midbrain following intraocular infection will be analysed more fully in a later paper, only a summary account being included here.

In a review of the lesions following intracerebral inoculation of active poliomyelitis virus Hurst (1929) states that in the midbrain perivascular infiltration was frequently intense in the floor of the fourth ventricle and in the substantia reticularis. Focal infiltration and small areas of diffuse infiltration occur, but are not as numerous as in the pons and medulla. Damage to nerve cells was rarely

severe, the signs being chromatolysis, irregular staining of cytoplasm and nuclear shrinkage, and actual necrosis was never seen. Brodie (1932), in general agreement, states that after intra- or extra-cerebral inoculation there was only an occasional neurone affected in the midbrain.

The pathological picture following intraocular infection presented a marked contrast to these findings. Instead of inflammatory changes in the substantia reticularis there were much more intense changes anterior to the iter of Sylvius and central grey substance, running forwards below the floor of the third ventricle. Not only were these changes usually acute, but destruction of nerve cells was extensive and neuronophagia proceeded actively. The region of the oculomotor nuclei was particularly involved. In some cases the attack was not of grave severity, and all the changes from chromatolysis, nuclear shrinkage and extrusion, to death of the cell could be seen. In others scarcely a nerve cell could be seen in the inflamed area. A dark patch covered by phagocytic microglial cells (Hurst, 1929) represented the end stage of the necrotic process. Tissue infiltration with many lymphocytes and swollen microglial cells, and perivascular cuffing were both very evident, and dilatation, proliferation and even budding of capillaries could be seen.

The changes elsewhere in the nervous system were generally less severe, and were of more recent origin than in the region of the oculomotor nuclei. The spinal cord from paralysed animals showed the usual intense damage to motor cells, but neuronophagia was not as advanced, and the compound granular corpuscles not so fully developed.

Monkeys killed on the day of onset of ptosis, before any paralysis of limbs became evident, exhibited intense, but variable, changes in the region of the midbrain described above. One monkey, killed on the eighth day, showed its first elevation of temperature to 105° F. and bilateral ptosis on the seventh day. The perivascular spaces extending in the midline from the aqueduct of Sylvius anteriorly to the interpeduncular fossa were distended with lymphocytes, although little neuronal damage was to be seen. There were no inflammatory changes in the cervical, thoracic or lumbar segments of the spinal cord.

Monkey 1 was killed on the fourth day when the temperature was first found to be elevated and when ptosis first became apparent. There was no weakness of limbs. The region of the oculomotor nuclei showed intense changes. The central parts were packed with lymphocytes, occasional polymorphs and swollen microglial cells. The neurones showed all stages of inflammation. In some the Nissl bodies had disappeared and the protoplasm stained irregularly. The nucleus was eccentric with a large deeply-staining nucleolus and a prominent nuclear membrane. Some of the cells, especially those situated peripherally, were shrunken, very irregular in shape, stained darkly, and no nucleus could be distinguished. At the periphery of the oculomotor nucleus some of the large cells showed fragmentation of the Nissl substance, appearing as a fine dust scattered throughout the swollen cell, and the nucleus was represented only by a lighter area containing a nucleolus. There was no accumulation of phagocytic cells around these neurones which had

apparently suffered recent acute mortal damage. Perivascular cuffing was not as intense in this monkey as in the previous one.

Virus reaching the brain from the posterior chamber of the eye would be likely to travel via the optic nerve, chiasma and tract to the lateral geniculate body and superior colliculus. Sabin and Olitsky (1938) have shown that this path is used when vesicular stomatitis virus is inoculated intraocularly in young mice. The only other axonal pathway which needs to be considered is via the long ciliary nerves and ciliary ganglion and the oculomotor nerves. Very little positive histological evidence could be obtained in favour of either route. Sections of the optic pathway showed very little change—at most small tissue infiltrations and a little perivascular cuffing in the lateral geniculate body. There were more marked changes in the superior colliculus as a rule. The occipital cortex showed no inflammatory changes. The ciliary ganglion in one case showed a little lymphocytic infiltration, extending through the capsule at the point of entry of the ciliary nerve.

Evidence of upward spread from the brain stem was seen in the hypothalamic nuclei and in the thalamus, but was most marked in the motor cortex, particularly in its lower part. This was the only part of the cerebral cortex affected. Here could be seen tissue infiltration and perivascular infiltration of vessels running to the surface, chromatolysis of Betz cells, and even neuronophagia of dead cells. The intense changes were almost confined to the actual layer of the Betz cells.

That the clinical syndrome was an incomplete third nerve palsy, involving the levator palpebrae superioris and the superior rectus on both sides, was surprising when the intensity and extent of the pathological lesion were seen. Impairment of lateral deviation was not seen, and only in one case was the pupil observed to be abnormal. This probably depends upon the fact that the lesion is most intense in the upper part of the oculomotor nucleus, for in this part are situated the cells supplying the levator of the upper lid and the upward rotators of the eyeballs.

DISCUSSION.

It is a well-known characteristic of the virus-antibody reaction that the apparent neutralizing activity of a given serum may vary widely according to the route by which the test inoculation is made. Most of the available data consists of comparisons between the intracerebral route and some other method, e.g. intradermal or intraperitoneal. Andrewes (1928), Sabin (1935), Fairbrother (1932) and others have shown that vaccinia virus-serum mixtures which are non-infective on intradermal injection may be almost fully active in inducing infection of rabbits by intracerebral inoculation. Sabin (1935) reports that whereas a pseudorabies antiserum is incapable of neutralizing a single lethal dose of virus if the test inoculation is made intracerebrally, many lethal doses may be rendered non-infective by the intranasal route. The results obtained when the same serum is titrated against poliomyelitis virus by both intracerebral and intraocular methods are clearly comparable to these findings with other viruses.

Our main object was to find the most suitable method for titrating successive samples of serum from individuals who had either suffered from clinical poliomyelitis or had been in family contact with such cases, and for such work the primary requirement was a method capable of showing up *differences* of antibody content as clearly as possible. An examination of Table 1 will show at once that the difference between sera 81 and 82 is much more clearly manifest by the intraocular method than by the intracerebral. The method has therefore been used throughout for the titrations of human sera to be reported in a subsequent paper.

Histological and experimental investigations on the route followed by the virus after intraocular inoculation are still incomplete. It seems highly probable that the virus deposited on the retina passes via the optic nerve, chiasma and tract to the lateral geniculate bodies and superior colliculus. The superior colliculus is concerned with reflex ocular adjustments, and has important connections with the oculomotor nuclei on both sides. It is by such connections that we must envisage the spread of virus to the oculomotor nuclei, with the consequent appearance of ptosis and of histological lesions. The possibility that the virus passes to this region by the ciliary ganglion and oculomotor nerves is not yet excluded. From the midbrain the virus passes down to the cord producing the characteristic lesions, and also to the motor cortex. The distribution of lesions is characteristic of poliomyelitis in being concentrated predominantly on motor cells. The virus passes almost silently over sensory pathways, but damages each set of motor cells that it meets, the oculomotor nuclei, the Betz cells of the lower motor cortex, and the anterior horn cells of the spinal cord.

SUMMARY.

Rhesus monkeys are highly susceptible to poliomyelitis virus (M.V. strain) by intraocular injection.

In a large proportion a bilateral ptosis is the initial symptom of infection.

Antibody titrations can be conveniently carried out by this route, and give higher titres and more regular results than with the customary intracerebral procedure.

A preliminary account of the pathological histology is given. Intense changes in the region of the oculomotor nuclei are the most characteristic feature.

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POLIOMYELITIS

2. THE SIGNIFICANCE OF NEUTRALIZING ANTIBODIES IN HUMAN SERA.

by

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The relative immunity of adults to poliomyelitis is probably regarded by most epidemiologists as a result of widespread subclinical immunization during childhood. The evidence for this theory is drawn from several sources. In the first place, the age incidence of the disease, affecting predominantly young children in cities, somewhat older groups in rural areas, and adults in isolated uncivilized communities, is difficult to interpret on any other hypothesis. Conclusive experimental evidence of the importance of subclinical immunization in regard to diphtheria has been provided during the last twenty years, and it is natural to assume that the other diseases of childhood should show essentially similar behaviour. In poliomyelitis technical difficulties make it impossible to detect carriers and abortive cases by bacteriological methods, so that the actual experimental evidence for subclinical immunization is confined to the results obtained in tests for the presence of neutralizing antibodies in human sera and the correlation of such results with the age of the subject, the urban or rural character of his environment, and any history of past infection or exposure to infection with the virus.

There is general agreement that most adult sera contain antibody capable of neutralizing poliomyelitis virus, and that the proportion of active sera increases with age during childhood. It has not, however, been shown at all satisfactorily that the appearance of antibody is a response to infection, clinical or subclinical, with the virus. Various workers have, in fact, shown that sera from patients recovered from paralytic poliomyelitis may be completely lacking in neutralizing antibody.

The earlier literature on the subject is reviewed in the Milbank Committee monograph (1932). Here we need only refer to the results collected from the literature by Harmon and Harkins (1936), which showed that of 183 convalescent sera 40 p.c. failed to neutralize the virus. As a control series they quote the results of examination of 512 "normal" sera. Divided into age groups, these normal sera gave the following figures: 0–4 years, 37 p.c. neutralized; 5–14 years, 48 p.c. neutralized; over 15 years, 73 p.c. neutralized.

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Even more convincing evidence of the lack of any relationship between clinical poliomyelitis and serum neutralizing antibodies, has been provided by comparative neutralization tests done on sera taken from patients at the onset of paralysis, and again some months later.

Harmon and his co-workers (1936) tested the sera from fourteen patients. Twelve out of the fourteen contained neutralizing substance at the onset of the disease. One patient, who later became paralysed, was bled in the pre-paralytic stage, and even at this early stage contained neutralizing antibodies. Of the fourteen sera taken some months later, only one failed to neutralize. Eleven of the pairs of sera were tested in more detail, using varying dilutions of sera, in an attempt to estimate their antibody titre. The results were somewhat irregular, but in only four of the eleven was there any indication of an actual increase in the antibody content of the serum between the first and second bleedings. These workers comment: "These observations . . . cast doubt upon the importance of a single attack of poliomyelitis for the production of antiviral substance".

It has been suggested by Trask and Paul (1933) that this failure to demonstrate the production of antibodies during an attack of poliomyelitis has been due to the use, by most workers, of a strain of virus which has undergone a long series of monkey passages such as the Rockefeller "M.V." strain. That strains of poliomyelitis may differ greatly in their virulence is well recognized. There is also evidence that minor but distinct antigenic differences may be recognized between strains (Burnet and Macnamara, 1929, Weyer, 1931, Flexner, 1932, Howitt, 1937, Kessel, Stimpert and Fisk, 1938, and several reports from Paul and Trask, 1933 and 1935). Trask and Paul (1933) present some experimental evidence that young children with poliomyelitis may develop antibody against a recently isolated strain, but not against a standard monkey passage strain. Howitt (1933) obtained a similar result with three cases, but both series are so small that the results cannot be regarded as more than slightly suggestive.

When the Victorian epidemic of 1937 was in progress, experimental studies were planned to determine definitely whether the passage of a typical, moderately severe epidemic through a city community did in fact result in the appearance of antibody in a considerable proportion of children who previously had no antibody. Arrangements were therefore made to obtain serum (a) from a series of infantile paralysis patients in the Fairfield Infectious Diseases Hospital at the time of admission with acute symptoms, and from the same patients two to three months later; (b) from contacts (brothers and sisters of patients), also taken at the time of primary infection in the family, and two to three months later. The sera were separated, ampouled and stored in the refrigerator until tested four to twelve months after being obtained.

At the time the investigation was planned we had not seen the papers by Brodie *et al.* (1937 and 1938), which cover very much the same ground, as far as the studies on patients' sera are concerned. They found that infection with poliomyelitis might occur in individuals with or without antibody, and that no significant change in the serological status of the individual took place within a year. Our observations are fully confirmatory of those made by the American workers, and for this reason a less extensive series of experiments was made than was originally planned.

The results obtained by Brodie and collaborators may be summarized as follows:

(a) Of 82 paralytic cases tested during the acute stage of the disease, the sera of 14 neutralized the virus;

(b) 4 of the 82 were tested in the pre-paralytic stage, and two of these possessed protective bodies;

(c) of 32 non-paralytic cases tested in the acute stage of the disease, the sera of 18 neutralized;

(d) during convalescence only two of 39 paralytics who had no protective substances in the acute stage developed them within a few months of the onset;

(e) neutralizing substances which had not been present in the acute stage in 11 non-paralytics also failed to develop in the sera tested several months after the onset;

(f) two paralytics and one non-paralytic, whose sera neutralized in the acute stage of the disease, failed to show a demonstrable increase of neutralizing substances two months or longer after the onset.

Using a recently isolated strain of virus, they obtained results comparable to those obtained with the passage strain.

They conclude that "no evidence of a definite relationship was found between the presence of protective substances in serum and resistance to poliomyelitis".

Our own experiments were made with a standard monkey passage strain (Rockefeller Institute, M.V.), and we used throughout the intraocular method previously described (Burnet, Jackson and Robertson, 1939). We have given evidence there that the method is particularly adapted to detect differences between a serum with no antibody and one containing only small amounts. It should therefore have provided a clear indication of the appearance of antibody after infection in a child previously lacking it. Despite the use of this technique, however, our results are in full agreement with those of Brodie *et al* in showing no significant appearance of antibody as a result of infection.

TECHNIQUE.

Young rhesus monkeys were used, and the serum titrations made by the intraocular method, which has been fully described in a previous paper. A 5 p.c. stock emulsion of virus in broth (prepared as described previously) was used throughout. 0·1 c.c. of a mixture of equal parts of stock emulsion and undiluted serum was used as the intraocular inoculum. In some cases various dilutions of serum in broth were used as well as undiluted serum. Each serum from the group of patients' sera was tested in at least two monkeys. During the course of the investigation two batches of stock virus showed an unsatisfactory degree of virulence by the intraocular route. They infected at a dilution of 1: 2, but only irregularly at 1: 20 instead of at 1: 200, as our first two batches had done. The sera tested at this period were therefore considered to contain no antibody if either one or both of two monkeys inoculated became paralysed. The sera which appeared to protect with this virus were all subsequently tested against a new stock of "M.V." prepared from the cords of infected cynomolgus monkeys. This infected three of four rhesus monkeys by intraocular inoculations at a dilution of 1: 100 and all at lower dilutions.

Tables 1 and 2 show the results of testing the sera of patients and contacts for the presence of antibody capabale of neutralizing the strain "M.V."

We may summarize these two tables as follows: Of fourteen patients with clinical poliomyelitis, five possessed antibody at the time of acute illness. The

TABLE 1.

Poliomyelitis Patients Tested Against "M.V."

Patient.	Age.	Dilution of serum.	First bleeding.	Second bleeding.	Interval between onset and first bleeding.	Interval between two bleedings.
W.B.	5	Undiluted	9, 10	15, (13)	1 day	2½ months
S.F.	6	„	7, ±	7, 8	2½ „	4 „
D.L.	7	„	8, 8	8, 12	2 „	2 „
P.L.	8	„	8, 8, 8	8, 8	3 „	2 „
D.J.	8	„	13, 8, 8	20, ±, 8	Pre-paralytic	3 „
R.R.	9	„	9, ±, 8, 8, 8	9, 9, 23, ±, 8	3 days	2 „
D.McD	9	„	±, 8, 8, 8	(18), 8, 8, 8	1 „	3 „
		1/10	±, 8	±, 8		
B.L.	9	Undiluted	±, 8	±, 8	?1 „	5 „
		1/10	±, 8	±, 8		
A.G.	10	Undiluted	7, 10	7, 8	12 hours	2 „
D.R.	11	„	12, (18), 8	9, 9, 8	2 days	4 „
J.L.	11	„	×, 8	±, 8	2 „	3 „
		1/10	8, 8	±, 8		
J.W.	11	Undiluted	7, 8	9, 9	5 „	4 „
J.T.	14	„	11, ×, 8, 8	33, ×, 8, 8	4 „	3 „
A.R.	18	„	8, 8, 8	8, 8, 8	3 „	4 „
		1/10	±, 8, 8, 8	8, 8, 8, 8		

Number = interval in days between inoculation and first paralysis.
8 = survived without signs of illness or paralysis for 21 days.
± = temperature > 104°F. without paralysis.
(18) = ? nonspecific death on eighteenth day.
× = nonspecific death.

TABLE 2.

Contacts Against "M.V."

Patient.	Age.	Serum dilution.	First bleeding.	Second bleeding.	Interval between two bleedings.
D.C.	5	Undiluted	12	7	4 months
W.C.	8	„	8	8	4 „
B.P.	3	„	8	8	1 „
W.P.	6	„	13	11	1 „
S.Q.	3	„	30, 16	11	1½ „
P.Q.	14	„	9, 12, 13, 8, 8	12	1½ „
J.T.	4	„	8	8, ±	1 „
M.T.	11	„	11, 21	13	1 „
C.S.	9	„	8	8	1 „
K.G.	11	„	8	8	1 „
E.S.	5	„	8	8	1 „
E.E.	6	„	9	10	1½ „
C.C.	10/12	„	8	10	1½ „
G.C.	12	„	8, 12	8, 10, ×	2 „
R.M.	9	„	12, 12	±, 7, 13	1½ „

other nine had insufficient antibody to neutralize under the conditions of our tests, and eight of these still failed to neutralize when convalescent. One, J.T., aged fourteen years, appeared on the result of a first test to have developed antibody during the interval between the two bleedings, but a repetition of the tests showed

that some antibody was also present at the time of first bleeding. Of fifteen contacts six possessed antibody, nine did not. The only discrepancy between first and second bleedings is the apparent disappearance of antibody in C.C., aged ten months. This might conceivably have meant the final loss of maternal immunity, but more probably signified merely unusual resistance to infection in the monkey used for the test of the first bleedings.

Neutralization Tests with a Local Strain of Virus.

In view of the evidence that poliomyelitis virus strains may show immunological differences, and that Trask and Paul (1933) have suggested that more "comprehensible" results of serum tests might be obtained by the use of early passage virus, we have carried out some experiments with a local strain of virus ("Mar"). This is less virulent than "M.V.", and rhesus monkeys which have recovered from paralysis with "Mar" almost always show a rise of temperature and sometimes an increase in paralysis when inoculated intracerebrally with "M.V." No deaths, however, have occurred in five monkeys previously paralyzed by "Mar". One monkey which showed only a rise in temperature after intraocular inoculation of "Mar" died typically after the test injection of "M.V." This is therefore the same sort of partial resistance as developed against "M.V." in our 1930 experiments, and may or may not be due to antigenic differences between the strains. The strain "Mar" is highly virulent for *M. cynomolgus*, and neutralization tests with it are most conveniently carried out in this species.

TABLE 3.

Comparative Titration of Pooled Convalescent Sera Against Passage and Local Strain of Virus.

Test.	Route.	Species.	S.82 + V.	S.82/5 + V.		
S.82 + M.V.	intracerebral	Rhesus	±, S	7, 8		
S.82 + M.V.	intraocular	„	±, ±, S	5, 9, ±		
S.82 + Mar (Cyn.)	intracerebral	„	7, 8			
S.82 + „ „	intraocular	„	6, S	5, 5		
S.82 + „ „	intraocular	Cynomolgus	7, 9			
			S.81 + V.	S.81/5 + V.	S.81/25 + V.	S.81/125 + V.
S.81 + M.V.	intraocular	Rhesus			S, S, S	S, S
S.81 + „	intracerebral	„		10, 13, S, S	8, 7, S	
S.81 + Mar (Cyn.)	intraocular	„		S, S	S, S	±, ±
S.81 + „ „	intracerebral	„	S, 16	S, 10	10, 10	
S.81 + „ „	intraocular	Cynomolgus	S, S	S, S	9, S	

In order to determine whether any antigenic differences between the strains "M.V." and "Mar" were of sufficient magnitude to influence our results with human sera, comparative titrations of two sera, S.81 and S.82, were made. These sera were both pooled sera from convalescents of the recent epidemic. S.81, from six individuals aged from six to twelve years, was known to contain active antibody against "M.V." If infection with local virus produces highly specific anti-

body not detectable in tests with the strain "M.V.", then the serum 82 should have been very suitable for its demonstration. Table 3 shows, however, a close agreement in the results with both viruses; if anything, there is rather less antibody against "Mar" than against "M.V." in serum 82. Serum 81 was obtained from convalescent patients, and it might be objected that antibody against "Mar" had developed in one or more of the patients after their recent infection. Serum A.R. (first bleed) was therefore tested against "Mar" virus in cynomolgus monkeys. Both monkeys remained well, indicating that even possession of antibody against the homologous strain may not be adequate to prevent paralytic infection.

DISCUSSION.

Examination of our protocols shows that in numerous instances dissimilar results were obtained in two monkeys inoculated with the same serum-virus mixture. We do not accept this as a criticism of our intraocular technique, as results just as irregular are obtained by the intracerebral technique. The importance of these discrepancies lies in the doubt they cast on reports of serum neutralization tests in which only one monkey for each mixture has been used. A critical examination of the protocols in the literature on poliomyelitis will reveal a very large number in which it is tacitly assumed that a given serum-virus mixture will always produce the same result when inoculated intracerebrally. In our experience this assumption is unjustified.

If, as in our experiments, the antibody contents of two successive samples of serum from each of a number of individuals are to be compared, it is essential that all should be tested. A fairly common procedure is to test the first samples using one monkey to each serum, and after dividing the individuals on these results into those initially with and without antibody, to test the second sample from those of the latter group only. It is obvious that if there are any irregularities in the results of the test they will all tend to suggest that a proportion of individuals have developed antibody between the two bleedings.

As regards the interpretation of our own results we feel that their significance is clear. Whilst individual sera may give discrepant results, it is striking that 27 out of the 28 pairs of sera show no indication of an increase in serum antibody as a result of (or concomitant with) infection or contact. Some of the children with paralytic poliomyelitis had antibody right at the onset of the disease, whilst others who had none at the onset still failed to neutralize some months later.

These results, taken along with those of Brodie *et al.* (1937), seem to be sufficiently definite to justify a discussion of their significance for the epidemiology of poliomyelitis. The facts may be summarized as follows: Children in the early stage of acute paralytic poliomyelitis may possess relatively large amounts of circulating antibody, or they may have none whatever. Those who have none do not develop any within two to three months, in our experience, nor within one year,

from the American work. Further, none of the family contacts who had no antibody at the time of contact developed it within a similar period. These tests were made with a standard monkey passage strain of virus, but a detailed study of two serum pools from convalescents in the same epidemic gave no indication that different results would be obtained with a strain of virus isolated from one of the fatal cases of the Victorian-Tasmanian epidemic.

It has been rather generally assumed that the antibody against poliomyelitis virus which is present in most adult sera results from subclinical infection with the virus at those times when the disease is prevalent in the community. Dudley (1929) showed clearly that when clinical diphtheria was present in a large naval school for boys there was an associated wave of subclinical immunizing infections capable of changing Schick-positive to Schick-negative reactors and this analogy has been applied to poliomyelitis. There is fairly substantial evidence that a smaller percentage of adults living in isolated country communities possess poliomyelitis antibody than is the case in urban populations (Aycock and Kramer, 1930), which is in agreement with the same conception. Our results, which are consistent with those of Brodie *et al.* (1937), and also with several previous smaller investigations, are completely opposed to such a view, unless we make the very unlikely assumption that antibody first appears more than a year after the immunizing infection. In monkeys recovering from experimental poliomyelitis antibody appears as a rule four to eight weeks after infection, and is of very much lower activity than average human adult serum. This is slower than the antibody response to many virus infections, but is much more rapid than the slow process which would have to be postulated for the human phenomenon. The only experimental data which offer any possible analogy are those obtained by Webster (1938) with mice immunized by an intraperitoneal inoculation of living St. Louis encephalitis virus. Immunity developed early, but antibody became detectable only eight to twelve weeks later, at a time when the immunity of the mice to intracerebral injection was waning or absent. Such a period in the life of a mouse might correspond to a year or two in man, based on rate of growth and senescence, but there is no evidence that immunological responses proceed at any faster rate in the mouse than in man. Further, the immunizing injection did not cause infection of the mouse central nervous system, and the conditions must therefore have differed profoundly from those in the human being infected with poliomyelitis.

We believe that the best interpretation of the facts is that in human beings the poliomyelitis virus infects only nervous tissues, and that in most cases very little virus or antigenic material derived from it passes to the antibody-producing cells of the body. There is therefore no significant appearance of circulating antibody in the great majority of instances. Since antibody obviously of specific origin can be obtained in monkey serum after infection, and since certain methods of human vaccination are reported to produce antibody in children, the possibility that a small proportion of individuals do produce antibody after poliomyelitis

must be left open, but such production cannot possibly account for the occurrence of relatively high titre antibody in a majority of human beings over ten years of age.

The orthodox view is that this antibody is a result of a process of widespread latent immunization by subclinical infections with the specific virus. There is no direct evidence whatever in favour of this hypothesis. It is undoubted that associated with every typical epidemic of poliomyelitis there are numerous recognizable but non-paralytic infections, and there is no logical reason why there should not be a very much larger number of completely unrecognizable infections as well. But no observer has shown that after the passage of an epidemic there has been a correlated increase in the number of children possessing antibody, and both Kramer and Aycock (1931) and ourselves find that there has been no such increase after an epidemic period. If immunization (in the sense of production of detectable antibody) does not occur at epidemic periods it seems extremely unlikely that it occurs at interepidemic periods, when only minimal numbers of paralytic infections are being observed. The fact that susceptibility to paralytic infection is only slightly correlated with absence of antibody in the serum is also indirect evidence that the antibody is not produced as a result of contact with the specific virus.

If we exclude specific immunization there seem to be three possible alternative origins of "poliomyelitis antibody" in human serum.

(1) It may develop as a result of a non-immunological change in serum globulin, which develops with age in the absence of antigenic stimulus.

(2) It may be the response to some commonly encountered antigen which has determinant groups in common with the poliomyelitis virus. The antigen must reach the antibody-producing cells in considerable amount, and hence is likely to be derived from a micro-organism which has some invasive power, but is not a strictly neurotropic virus.

(3) The antibody is a non-specific or group antibody, appearing as a result of repeated infections with various micro-organisms, not necessarily related antigenically to poliomyelitis virus.

Of these alternatives we may fairly confidently exclude the first "maturation" hypothesis on account of the wide individual variation in the age at which antibody appears in the blood. The one example of what might be called a "maturation" antibody known to us is that against the swine influenza virus. This appears with considerable regularity at about the age of ten years in those individuals who have been infected with any strain of influenza virus, but not in those who have never encountered the virus. It is not therefore, strictly speaking, a maturation antibody, but there is no doubt that a change in the quality of influenza antibodies occurs at about the age of ten to twelve years. Poliomyelitis antibody, however, like diphtheria antitoxin, may be present or absent at any age, and its appearance seems likewise to be determined by irregularly occurring environmental factors rather than by any intrinsic maturation. The same argument may be applied to

favour the second alternative rather than the third, but the decision could only be made by the accumulation of further knowledge. As far as we are aware, there is no fully substantiated record in the literature of an individual whose serum at first containing no poliomyelitis antibody later developed it in adequately measured amount, and therefore no indication of the nature of the event responsible for the change. If it could be shown by careful test of serum samples from the same children over a period of years that poliomyelitis antibody made a sudden appearance, one could be certain that a definite antigenic stimulus was concerned. If, on the other hand, there was a gradual development over a period of years, the third alternative above would clearly be more acceptable. On the evidence available we should be inclined to favour the second alternative, and look for the antigenic stimulus amongst the micro-organisms (bacteria or viruses) which produce common minor infections.

SUMMARY.

The antibody content of sera from poliomyelitis patients taken at the acute stage has been compared with that of sera obtained from the same patients two to three months later. In 14 cases no evidence of significant change in antibody titre was observed.

Similar tests of first and second bleeds from 13 brother and sister contacts of poliomyelitis cases also showed no appearance of antibody between the two bleeds.

Five poliomyelitis patients possessed active circulating antibody at the time of acute illness.

It is concluded that "poliomyelitis antibody" is not a result of exposure to or infection by the virus of epidemic poliomyelitis.

ACKNOWLEDGMENT.

We wish to express our gratitude to Dr. Stanley W. Williams, not only for his indispensable work in collecting the sera used in this investigation, but also for his help in obtaining clinical notes, tracing convalescent patients, and acting as general liaison officer between the laboratory and the hospitals.

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THE EFFECT OF SEVERE HAEMORRHAGE ON THE CONCENTRATION OF COPPER IN THE BLOOD OF SHEEP

by

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From time to time, since the recognition of the importance of copper in haematopoiesis, claims have been made that blood copper rises after severe haemorrhage.

Thus, Sarata and Suzuki (1933), who worked with rabbits, report that after drawing 17–23 ml. of blood per kilo. of body weight there was a two- to a six-fold rise in blood copper; the maximum always occurred on the first day following haemorrhage, after which it fell gradually to normal. Warburg and Krebs (1927) previously found a three- to a six-fold increase in serum copper after haemorrhage in the pigeon and goose. Potter, Elvehjem and Hart (1938) in a general paper quote Sachs (1938) in stating "it is known that haemorrhage mobilizes copper into the blood stream". This paper is not available in Australia, but from the context it is assumed that Sachs worked with dogs.

On the other hand, Guillemet (1932) has claimed that, in the dog, blood copper is considerably reduced after repeated bleedings, and Locke, Main and Rosbash (1932) have reported a similar effect in the rabbit after haemorrhage. Sarata and Suzuki suggest that this inconsistency may "depend on the method of producing anaemia or of determining copper", but we consider that for comparative purposes the methods for copper determination would be adequate.

Potter *et al.* suggest that "the theory that copper reserves are mobilized into the blood whenever haematopoiesis occurs" may be tentatively accepted.

The purpose of the present paper is to show that in sheep the loss of 25 ml. of blood per kilo. body weight is not associated with any disturbance of the concentration of copper in whole blood.

METHODS OF ANALYSIS.

Blood Copper. The method used is essentially that of Tompsett (1934). The blood is collected from the jugular vein of the sheep into a well oiled (paraffin oil), all-glass syringe which

has been calibrated between two graduation marks (say 5 and 15 ml.) to deliver an exact amount of blood (about 10 ml.). The sample is run directly into 30 ml. of glass-distilled water in a 100 ml. conical flask. The flask is shaken immediately to prevent clotting and to ensure complete lysis of the cells. 10 ml. of 40 p.c. trichloracetic acid are added to the flask, which is thoroughly shaken and the contents filtered through a washed, Whatman No. 1, filter paper. To 25 ml. of the filtrate (or smaller quantities where necessary) in a 50 ml. stoppered cylinder, are added 2 ml. of 4 p.c. sodium pyrophosphate, 1–2 drops of cresol red indicator and ammonia (S.G. 0·880) till just alkaline. Then 0·5 ml. of ammonia in excess, 10 ml. of amyl alcohol (B.P. 130–132°C.) and 1 ml. of 0·5 p.c. aqueous solution of sodium diethyldithiocarbamate are added. The cylinder is stoppered and thoroughly shaken, allowed to stand and the amyl alcohol layer drawn off and centrifuged for a few minutes to free it from water. The alcoholic solution is compared in the colorimeter with a suitable standard, prepared in the same way as the sample to be tested from a stock solution of copper sulphate containing 0·1 mg. Cu/100 ml.

It is essential that all filter papers be washed with dilute nitric acid and glass-distilled water and dried before use. We have found no filter papers free from copper and all have had to be washed.

The colour developed is not stable in amyl alcohols boiling below 130–132°C.

Haemoglobin. The acid haematin method, with the Leitz Sahli Haemometer, has been used throughout, with a 3-minute interval between acidification of the blood and reading. This method is regarded as sufficiently accurate for comparative purposes.

EXPERIMENTAL.

Experiment 1. Aged ewes, which had been on pasture for the preceding nine months, were placed in stalls and fed on oaten chaff and green lucerne. From five of these sheep blood samples were taken daily for five days for the determination of the copper and haemoglobin content. On the sixth day blood was drawn through a cannula from the jugular vein of each at the rate of from 6 to 15 ml. per kilo. body weight. Daily blood copper and haemoglobin estimations were continued for a further five days. The first estimations were made six hours after bleeding, the second 24 hours, and from then on at 24-hour intervals. During the next few days haemoglobin values fell, but only slightly. The greatest loss was in one sheep from which 15 ml. blood/kilo. had been taken, the haemoglobin figure (Sahli) falling by only 22 p.c. after 48 hours. Throughout the whole period of the experiment blood copper values remained normal (0·10–0·15 mg. Cu/100 ml.).

Experiment 2. This experiment aimed at producing a more severe anaemia in the experimental animals. Five sheep ranging in weight from 54 to 64 kilograms were stalled and blood drawn from each at the rate of 25 ml. per kilo. body weight (i.e. 1350–1600 ml. blood). Details of sampling and bleeding were the same as in the first experiment.

Fairly severe anaemia resulted in these sheep, but again there was no effect on the blood copper values.

The records of haemoglobin and blood copper values for these animals are shown graphically in the accompanying figure; arrows indicate the day of bleeding.

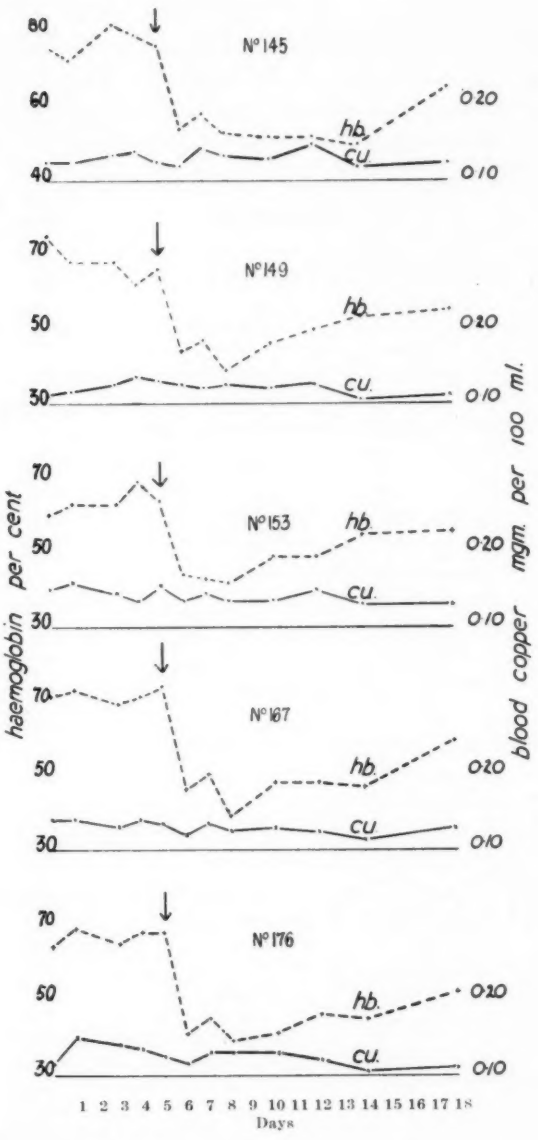

Fig. 1. Haemoglobin and blood copper records of sheep in Experiment 2. Arrows indicate the time of drawing 25 ml. of blood per kilo. body weight.

SUMMARY.

Previous workers have found that after severe haemorrhage in the rabbit, pigeon, goose and dog the concentration of copper in the blood rises several fold.

In the sheep we have found that the anaemia resulting from the loss of large volumes of blood (up to 25 ml. of blood/kilo. body weight) is not followed by any disturbance of the concentration of copper in the blood.

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PASSIVE IMMUNITY IN EXPERIMENTAL PERTUSSIS

by

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INTRODUCTION.

Most investigators of prophylactic vaccination against whooping cough have stressed the need for some laboratory test to detect the appearance and duration of immunity in vaccinated children. Although *H. Pertussis,* in large doses, is lethal to various laboratory animals, and can produce erythema or necrosis in the skin of the rabbit, no neutralization of the endotoxin responsible for these effects is obtained with immune sera of animals, or of patients convalescent from whooping cough (Evans and Maitland, 1937, 1939). Burnet and Timmins (1937) showed that the mouse was susceptible to infection by *H. Pertussis* given intranasally, and was protected against infection by prior immunization with living or heat-killed organisms given intraperitoneally. They suggested that the method might prove convenient for study of the antigenic qualities of various types of vaccines, and it has been since used for that purpose (Cruickshank and Freeman, 1939). We commenced our studies on these lines, but found that direct immunization of the mice had some disadvantages, particularly the length of time required for the completion of experiments and the consequent risk of non-specific deaths from intercurrent disease. We were led to test, therefore, the possibility of passively protecting mice against infection. Fortunately, the first samples of sera, obtained from adult contacts of whooping cough patients, were highly protective; more so, in fact, than any subsequently encountered, and we proceeded with the use of this method. It has the advantage that the state of immunity of human subjects can be directly investigated.

METHODS.

The method of inoculation of mice has been described fully by Burnet and Timmins (1937). Two criteria of the protective power of sera have been used.

(1) Reduction of mortality in mice given 50 million organisms intranasally. This dose kills 80–100 p.c. of controls with regularity.

(2) Prevention of lung infection in mice given 5,000 organisms intranasally, a dose producing a non-lethal infection in approximately 80 p.c. of controls.

The results of both types of experiment have been evaluated statistically.

We have not used, as an index of protection, the extent of pneumonic consolidation, since it is our experience and that of others (Burnet and Timmins, 1937) that large numbers of organisms may be recovered in the absence of detectable macroscopic evidence of infection. Consolidation or engorgement was invariably less marked or absent in the lungs of protected mice.

Sera.

Serum was given intraperitoneally, usually 4 hours, occasionally 18 hours before the infecting dose. In routine experiments it was given, irrespective of potency, in a dose of 0·30 c.c. or 0·25 c.c.

The sera used were from the following sources:

1. *Human Sera.*

(a) *Adult contacts.* Obtained in 1937, during an epidemic, from mothers of children with whooping cough, proved bacteriologically. Most of the women had symptoms of mild infection with *H. Pertussis*, but a positive cough plate was obtained from only one of them.

(b) *Normal adults.* Pooled sera of healthy young adult males, with no history of recent contact with whooping cough.

(c) *Convalescent.* From children with whooping cough, proved bacteriologically, taken 5–6 weeks after the appearance of symptoms. One pooled sample was obtained in the 1937 epidemic.

(d) *Immunized children.* Sera from children immunized 6–9 months previously with 80,000 millions of a vaccine prepared from freshly isolated Phase I strains.

(e) *Normal children.* Sera from children in the same age group as the immunized children. Care was taken to exclude children who gave histories suspicious of whooping cough.

2. *Rabbit Sera.*

Prepared by immunization of rabbits with living cultures of Phase I and Phase III strains, in 5 injections over a 3-week period, the total dosage being 78,000 million organisms, bled 7 days after the last injection.

No preservative was added to either human or rabbit sera. *Agglutination tests* were carried out in Dreyer's tubes in a water-bath at 50°C.

Examination of lungs.

The lungs of survivors in experiments using the lethal dose method were usually cultured, as were, of course, the lungs of all mice in experiments in which reduction in intensity of infection was the criterion of protection. The cut surface of the lower lobe of each lung was rubbed on a Bordet-Gengou plate. The degree of infection was graded according to an arbitrary scale in which:

++++ = confluent growth (> 1,000 colonies per half plate)
+++ = semi-confluent growth (circa 500 colonies per half plate)
++ = numerous discrete colonies (> 10 < 100 colonies per half plate)
+ = occasional colony (< 10 colonies per half plate)

When the growth from each lung differed, the higher figure was taken as the index of infection. This occurred only occasionally, usually in light or very light infections.

3. *Strains of H. Pertussis.*

All strains have been maintained on Bordet-Gengou medium containing 33 p.c. defibrinated horse blood, and have been sub-cultured weekly.

The smooth strains used in the experiments were recently isolated from children with clinical pertussis. They have been checked repeatedly during the period of use for Phase I characteristics.

Other strains isolated from one to three years previously, which are still agglutinated to high titre by sera prepared from smooth strains but which are also agglutinated to a greater or less degree by sera prepared from the rough strain "Hektoen", in comparative tests were as virulent as freshly isolated strains.

The rough strain "Hektoen" was originally obtained from the National Collection of Type Cultures in 1929, and has been maintained at these Laboratories on serum agar. During these experiments it was sub-cultured weekly on Bordet-Gengou medium. It is probably in Phase III of Leslie and Gardner. It is not agglutinated by sera prepared with freshly isolated strains, but sera prepared from it agglutinate freshly isolated strains in low dilutions. The individual organisms are definitely bacillary, about 2–4μ long. Capsules can be demonstrated. The colonies on Bordet-Gengou medium are much larger than those of freshly isolated strains.

Smooth strains of *H. Pertussis* multiply in the mouse lung following intranasal inoculation of as few as 50 organisms.

The rough strain was non-lethal for the mouse in large doses (5,000 million organisms) by the intranasal route. The organisms are rapidly cleared from the lungs. The virulence could not be increased by mouse passage.

4. *Preparation of Suspensions.*

The growth from a twenty-four-hour culture on a Bordet-Gengou slope was emulsified in nutrient broth and the bacterial content estimated by comparison with standard opacity tubes. The requisite dilutions were also made in nutrient broth. Colony counts of suitable dilutions on Bordet-Gengou medium gave figures which corresponded sufficiently closely to the estimated bacterial content for our purposes in these experiments.

RESULTS.

Protection with Sera of Adult Contacts.

The findings with a highly potent serum, AC 8, used as a reference standard throughout the work, will be described first. The action of a constant dose of 0·25 c.c. of this serum on mice infected with graded doses of *H. Pertussis* is shown in Table 1, that of graded doses of the serum against a constant dose of organisms in Table 2. The protective action of this serum against the lethal effects of *H. Pertussis* infection is obvious. This effect is rapidly lost on reducing the dose of serum. The full action is obtained with 0·3 c.c. Increasing the dose to 1·0 c.c. did not reduce the mortality, while a higher percentage of the lung cultures of survivors were positive. This paradoxical action is possibly due to the depressant effect on the mouse of large amounts of foreign serum. That the serum has an anti-bacterial action is shown by the fact that the lungs of all mice given 0·3 c.c. were sterile.

We suspected that there might be an antitoxic element in the action of this serum, because serum-treated mice looked healthy 18 hours after inoculation with 50 million organisms, in striking contrast to the unprotected controls. The serum, however, does not protect mice against one lethal dose of pertussis endotoxin, nor has it any detectable neutralizing effect on the necrosis following intradermal in-

jection of endotoxin in the rabbit. Unless there is an undetected exotoxin of *H. Pertussis*, we must assume that the action is solely directed against the multiplication of the organisms.

TABLE 1.

Protective Action of Serum from Adult Contact of Whooping Cough against Lethal Action of H. Pertussis.

Constant dose of serum: Graded doses of *H. Pertussis*.

Dose of *H. Pertussis*.	Serum Treated. Day of Death.	Number surviving.	Controls. Day of Death.	Number surviving.
50,000,000	2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 15	10/20	1, 1, 1, 1, 1, 1, 2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 4, 4, 8	0/20
5,000,000	7, 8, 12	17/20	3, 4, 4, 5, 5, 5, 6, 6, 9, 11, 12, 13, 14, 14, 14, 14, 20, 21	1/20
500,000		20/20	8, 8, 9, 10, 15, 19, 20, 20, 20	11/20
50,000	6	19/20	14	19/20
5,000		20/20	14	19/20

0·25 c.c. of serum AC 8 given intraperitoneally 4 hours prior to test.
Controls given no serum.
Mice observed for 3 weeks.

TABLE 2.

Protective Action of Serum from Adult Contact of Whooping Cough Compared with Pooled Serum of Healthy Adults.

Dose of Serum.		No. of mice dead.	Day of death.	Intensity of infection of survivor's lungs.
AC 8	1·00 c.c.	2/10	2, 3	++++, ++, ++, +, +, 0, 0, 0
AC 8	0·30 c.c.	2/10	3, 5	0, 0, 0, 0, 0, 0, 0, 0
AC 8	0·10 c.c.	6/10	1, 2, 3, 3, 3, 7	++++, +++, ++, +
AC 8	0·03 c.c.	9/10	1, 1, 2, 2, 2, 2, 2, 2, 2, 3	++++
N.A.S. (a)	0·30 c.c.	8/10	1, 2, 2, 2, 2, 2, 3, 3	++++, ++++
N.A.S. (b)	0·30 c.c.	9/10	2, 2, 2, 2, 2, 3, 3, 3, 3	++++
No serum		10/10	1, 2, 2, 2, 2, 2, 2, 2, 2, 2	

Mice given 50 million *H. Pertussis* intranasally.
Survivors killed 7 days after inoculation and lungs cultured.
A.C. = serum of adult contact.
N.A.S. = pooled serum of healthy adult non-contacts (a and b are different samples).

We have tested several other samples of "adult contact" sera. Most of them showed a definite protective action, whereas "normal" adult serum had no such effect. In a typical experiment, employing groups of ten mice, of the controls (no serum) 8 died; of those given "normal adult" serum, 9 died; of four groups given different specimens of "adult contact" serum, 4, 5, 5 and 1 died, the last figure being that for the reference serum, AC 8. In a large number of experiments we have never found less than 8 of 10 mice die when inoculated with a dose of 50 million organisms. Whereas in this experiment the lungs of the survivors in the group given the highly protective serum, AC 8, were sterile, those of survivors in the groups given the less active sera gave confluent or semi-confluent growths.

TABLE 3.

The Effect of Pooled Convalescent Children's Serum on the Intensity of Infection with H. Pertussis.

		Intensity of Mouse Lung Infection.	No. of lungs specifically infected.
Convalescent Serum (11 pooled samples)	0·3 c.c.	++++, +, +, +, +	5/18
Pooled normal infants' serum	0·3 c.c.	++++, ++++, ++++, ++++, ++++, ++++, ++++, +++, +++, +++, +++, ++, ++, +, +	15/19
Controls (no serum)		++++, ++++, ++++, +++, +++, ++, ++, ++, ++, ++,	10/10

The difference between the two groups given serum is statistically significant ($n = 1$ $\times^2 = 9{\cdot}7$ $\rho < {\cdot}01$).

TABLE 4.

Protective Action of Pooled Sera of Children Immunized Against H. Pertussis.

Serum.	Dose.	No. of Deaths.	Day of Death.	Intensity of infection of survivor's lungs.
Pooled, Immunized, Children's	1·0 c.c.	4/10	3, 4, 4, 5	++++, ++++, +++, +++, +++, 0, ...
" " "	0·3 c.c.	4/10	2, 2, 2, 4	++++, ++++, ++++, ++++, +++, +++, ...
Normal Children's	1·0 c.c.	8/10	2, 2, 2, 2, 2, 2, 3, 3	++++, ++++, ...
" "	0·3 c.c.	9/10	2, 2, 2, 2, 3, 3, 6, 6, 6	++++, ...
Reference Serum (AC 8)	0·3 c.c.	2/10	5, 5	++, ++, +, +, 0, 0, 0, 0, . .
	0·1 c.c.	3/10	2, 4, 6	+++, +++, ++, ++, +, +, 0, . . .
No Serum (controls)		10/10	2, 2, 2, 2, 2, 3, 5, 6, 6, 6, 7	

Dose of *H. Pertussis* 50×10^6 organisms.
Mice observed 7 days and lungs of survivors cultured.
The difference between the total mortality (8/20) in the mice receiving immunized children's serum and that (17/20) in those receiving the serum of control children is significant ($n = 1$ $\times^2 = 8{\cdot}6$ $\rho < {\cdot}01$).

Results with Sera from Patients Convalescent from Whooping-cough.

Unfortunately, at least from our point of view, there have been very few cases of whooping cough in Melbourne during the last year. We have had few opportunities therefore of testing convalescent sera. It is difficult also to obtain from young children the minimum amount of blood (15 c.c.) required for adequate tests. Using the lethal protection test, the serum of one child taken in the sixth week was highly protective (6 of 6 mice surviving), while that of 7 others taken in the fifth week showed no striking protective action. At the time when these tests were made we had not developed the technique of demonstrating protective antibody by using small, non-lethal doses of organisms. Tested by this method, a sample of pooled convalescent serum was shown to be significantly protective. The data are shown in Table III.

The Protective Effect of the Sera of Immunized Children.

In our early tests with the sera of immunized children, using the lethal dose method, our results were irregular, though in one experiment (Table 4) a significant protective action was noted. Since the excellent studies of various American workers, notably Sauer and Kendrick, indicated unmistakably that immunized children possess a substantial resistance to the natural disease, it was felt that the lethal test might be too exacting. It was decided to see whether such sera would protect mice against infection by a small dose, 5,000 organisms, which we had shown previously would infect about 80 p.c. of mice. The result of such an experiment is shown in Table 5, and it is seen that the serum of children immunized with Phase I vaccine will protect mice against infection with *H. Pertussis*. The pooled sera of immunized children were markedly less protective than the best of the "adult contact" sera.

The Protective Effect of Rabbit Sera.

In several tests, using the lethal dose method, sera from rabbits immunized with living Phase I cultures, agglutinating freshly-isolated strains in 1:10,000 dilution, failed to protect mice. One of these Phase I sera was tested therefore by the more sensitive technique, using as controls groups of mice given Phase III serum and normal rabbit serum. Tested in this manner, the Phase I serum showed a significant protection; but, somewhat unexpectedly, the Phase III serum was equally effective. The experiment was repeated, using different specimens of Phase I, Phase III and normal rabbit sera, with practically identical results, which are statistically highly significant. The data are shown in Table 6. We can conclude that both Phase I and Phase III rabbit sera, prepared with virulent and avirulent cultures respectively, can both passively protect mice against infection with *H. Pertussis*. There is no significant difference in the protective power of the sera prepared from virulent (Phase I) and avirulent (Phase III) cultures.

TABLE 5.

The Effect of Immunized Children's Serum on the Intensity of Infection with H. Pertussis.

	Intensity of lung infection.	Nos. of lungs specifically infected.
Immunized children's serum 0·3 c.c.	++, ++, ++, +, +	5/20
Controls (no serum)	++++, ++++, ++++, ++++, +++, +++, +++, +++, +++, +++, +++, +++, ++, ++	14/28

Dose 5,000 organisms. Mice killed on seventh day. The lung cultures of 4 mice in the control group showed heavy contamination. The results from these mice were discarded.

The difference in the percentage of lungs infected in the groups is statistically significant ($n = 1$ $\times^2 = 10{\cdot}55$ $p < 1/1{,}000$).

Note also that whereas the infections in the control group were mainly heavy, those in the protected group were very light.

TABLE 6.

Protective Action of Serum from Rabbits Immunized with H. Pertussis, *Phase I and Phase III.*

	Intensity of lung infection.	Nos. of lungs specifically infected.
Phase I (a)	+++, +++, +, +, +, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	5/20
Phase III (a)	++, +, +, +, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, C, C, C, C	4/16
Normal Rabbit Serum (a)	+++, +++, +++, +++, +++, +++, ++, ++, ++, ++, +, +, +, 0, 0, 0, 0, 0, 0, C	13/19
Phase I (b)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0/16
Phase III (b)	++++, +++, +++, +, 0, 0, 0, 0, 0, 0, 0, 0, 0, C, C	4/14
No serum (b)	++++, ++++, +++, +++, +++, +++, ++, ++, ++, +, +, +, 0, 0, 0, C	12/15

C = contaminated lung culture, result discarded.

Dose 5,000 organisms. Mice killed on 7th day.

(a) and (b) indicate separate experiments with different samples of sera.

In these experiments, separately and combined, Phase I and Phase III sera show a significant protective effect as compared with the controls, but no significant difference when compared with each other.

Phase I	v. controls (a)	$\times^2 = 7{\cdot}4$ $p < {\cdot}008$	(b)	$\times^2 = 19$ $p < {\cdot}0001$
Phase III	v. controls (a)	$\times^2 = 6{\cdot}4$ $p < {\cdot}01$	(b)	$\times^2 = 7{\cdot}7$ $p < {\cdot}006$
Phase I	v. Phase III (a)	$\times^2 = 0$	(b)	$\times^2 = 4{\cdot}6$ $p < {\cdot}035$

The Lack of Correlation between Protective Action of Sera and Agglutinin Titre.

All samples of sera were tested for their agglutinin titre against Phase I suspensions. There is no correlation between the protective antibody content of the sera and their Phase I agglutination titres. The best protective serum (AC 8) has a very low agglutinin titre (1:10), while the poorly protective sera from immunized children have a relatively high agglutinin titre (1:80). The Phase I rabbit sera which agglutinate Phase I organisms at 1:10,000 have only a weak protective action. Studies in progress by one of us (G.A.) indicate that more than one agglutinogen/agglutinin system is involved in the usual Phase I agglutination test, and it is hoped that analysis of the factors concerned may throw light on the antigen responsible for the development of the protective antibody.

DISCUSSION.

There is no reasonable doubt that the protective action which has been described is the result of the development of a specific anti-bacterial antibody in the serum following natural infection with *H. Pertussis* or artificial immunization. The protective antibody is not present in the sera of non-immunized children who have not had whooping cough, but appears following prophylactic immunization. It was present in the sera of most of the adult contacts of patients with whooping cough, following symptoms suggestive of mild infection, but not in healthy young adults who were non-contacts. It appears in rabbits in response to inoculation of living cultures of *H. Pertussis*. It must be admitted, however, that a limited number of attempts to absorb the protective fraction from the most potent human sera with both living organisms and the endotoxin were inconclusive. The specificity of the protection, and also the existence of an antibody not disclosed by agglutination, is indicated by the fact that mice injected with potent human sera of very low agglutinin titre and subsequently with pertussis endotoxin show immediate signs and symptoms of anaphylactic shock, which do not appear when endotoxin alone is injected, nor in mice sensitized with the rabbit sera we employed. Similar anaphylactic effects with rabbit sera have been described in the guinea-pig (Evans and Maitland, 1937), and it is therefore possible that our rabbit sera are less active than the samples used by them.

The findings indicate that inoculation of a living culture of a rough avirulent strain of *H. Pertussis* provoked the appearance of protective antibody in the rabbit. We did not detect any highly significant difference between the protective potency of the sera of rabbits immunized with smooth virulent (Phase I) and rough avirulent (Phase III) strains. We do not assert that both types of strains should provide equally efficient antigens for prophylactic immunization. The rabbits were given much larger doses proportionately than are used in children, so the essential immunizing antigen might be present in smaller amounts in the rough strains, or

might not be identical in constitution with that of smooth strains. Further investigations are in progress to determine whether vaccines prepared from avirulent strains can cause the appearance of protective antibody in children. The lack of correlation between the Phase I agglutinin titre and the protective antibody content of the sera establishes what has long been suspected, that the agglutinin titre of vaccinated children's serum is not a dependable index of immunity.

The methods described are the only ones available for direct demonstration of protective antibodies in human sera. Nevertheless, they are not without disadvantages. To obtain significant results with the majority of sera, large numbers of mice, large volumes of serum, and large quantities of media are required. Where it is permissible to pool serum, as in the demonstration of the development of immunity in a group of vaccinated children, these objections are largely overcome, but testing of the serum of individuals is hardly practicable except to settle points of theoretical importance. Pooling of sera may also lead to erroneous results, for doses of less than 0·1–0·2 c.c. of the most potent sera are relatively ineffective, so that dilution of a highly active serum by three or four inactive samples would lead to a negative result.

It may be questioned whether children whose sera show only a weak effect against such a small dose as 5,000 organisms are likely to be highly resistant to pertussis. In the first place the concentration of antibody in the mouse cannot be more than a fraction, at best one-hundredth, of its concentration in the subject from which it was derived. Secondly, it is a foreign serum. Thirdly, the mouse is exposed to infection when anaesthetized, so that the organisms are directly inhaled into the lungs. As a point of curious interest, but probably of no significance, the number of infections in mice protected with immunized children's serum (25 p.c.), as compared with controls (80 p.c.), is very close to the figures found for immunized and non-immunized children exposed to the natural infection (Kendrick, 1938; Singer-Brooks, 1938; Miller and Faber, 1939). Finally, the immunity of children recovered from the natural disease can be broken down readily under conditions of prolonged and intense exposure to infection, as in institutional outbreaks of whooping cough (Faber, 1938).

We think it highly probable, therefore, that the presence of circulating protective antibody plays an important part in the resistance of children immune to pertussis as a result of the natural disease or prophylactic vaccination.

SUMMARY.

Methods are described for the demonstration of antibody protecting mice against infection with *H. Pertussis*, injected intranasally under anaesthesia.

This protective antibody is shown to be present in the serum of some adult contacts of whooping cough, of children convalescent from whooping cough, of

children immunized with *H. Pertussis* (Phase I), and in rabbits immunized with living cultures of *H. Pertussis*, both virulent (Phase I) and avirulent (Phase III) strains. It is not detectable in pooled serum of healthy adults, or in the serum of children who have not had whooping cough, and who have not been immunized.

The protective antibody is antibacterial in its action.

There is no correlation between Phase I agglutinin titre and the protective potency of sera.

The significance of these findings is discussed, and it is concluded that this antibody plays an important part in the resistance to whooping cough of children who have recovered from the disease or who have been prophylactically immunized.

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THE EFFECTS OF VARIATION IN NITROGEN-SUPPLY AND WATER CONTENT ON THE AMOUNTS OF CARBOHYDRATES IN THE LEAVES OF GRASS PLANTS

by

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INTRODUCTION.

In earlier papers from this Laboratory there have been presented the results of experiments designed to investigate the effects of varying water-content and of ammonium salts supplied as a nutrient on the amounts of substances participating in reactions comprising the nitrogen and sulphur metabolism of the leaves of grass plants (Petrie and Wood, 1938a and b; Wood and Petrie, 1938; Wood and Barrien, 1939a and b; Barrien and Wood, 1939).

In this paper we present the results of an experiment which extends our observations to include various carbohydrates in the leaves, and consideration is given to relations existing among carbohydrates when water and also synthesis of organic-nitrogen compounds vary in amount. The experiment was of an exploratory kind. From consideration of the data certain significant relations between variables have been established; while in other instances relations, whilst they cannot be shown to be statistically significant, are nevertheless suggestive of a real effect. We have utilized all these data in an endeavour to establish the most likely course of the reactions comprising the carbohydrate and nitrogen metabolism of the plant.

In its essentials the experimental method employed was that used in the above-mentioned work (*loc. cit.*) and consisted in growing plants of a pure line of grass in washed quartz sand in pots. Up to the experimental stage all pots received the same amount of nutrients. At the experimental stage the pots were placed in a random manner in an environment constant in certain respects, and after the lapse of a definite time certain factors (in the present case light intensity and nitrogen supply) were varied. After the lapse of a further period of time appropriate pots were removed at intervals for analysis.

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By making as many factors as possible constant it was hoped that the reactions comprising the metabolism of the plant might approximate to a drifting steady state, consideration of which has been discussed by Petrie and Wood (1938a). A steady state was not expected and could probably only be realized by enclosing isolated plants under constant environmental conditions, adjusting the light to the compensation point, and also adjusting the ammonia supply to such a low level that no further synthesis of nitrogen occurred. Actually, as will be shown in the subsequent sections, the system as a whole drifted with time but there is evidence that it was not far removed from a steady state.

Several workers have investigated phases of the problem outlined above, although in no case have all the carbohydrate fractions been estimated nor have attempts been made to control the external environment. These investigations fall roughly into two groups; first, those concerned with the inter-relations of the carbohydrate and nitrogen metabolic cycles especially in relation to respiration; and second, those concerned with the effects of change in water content on the amounts of various carbohydrates.

Gregory and Baptiste (1936) determined total sugars in successive leaves on the stem in barley plants rendered deficient to differing degrees in nitrogen, potassium and phosphorus. In general, high total reducing sugars were associated with low respiration rate and low protein synthesis, whilst low sugars were associated with high respiration rate, high protein synthesis and low assimilation rate. Gregory and Sen (1937) used the same experimental technique and estimated sucrose and reducing sugars in barley leaves. Except in some of the potassium deficient plants amount of protein was significantly correlated with respiration rate; low respiration rate accompanied high sugar in nitrogen deficient plants and high respiration rate accompanied low sugar in potassium deficient plants. They have formulated a scheme showing the inter-relations of carbohydrate and nitrogen metabolism and suggest that carbohydrate oxidation is regulated by the rate of protein synthesis.

Archbold (1938b) investigated in barley the seasonal drifts of amounts of fructosan, sucrose and reducing sugar and discusses the inter-relations between them. She found that high level of nitrogen manuring reduced the amount of fructosan and considered fructosan formation as a relatively slow process taking place at the expense of invert sugar produced from sucrose and entailing the inter-conversion of glucose and fructose. Sucrose was considered to be the starting point for polysaccharide synthesis and she suggested that fructose was the source of carbon for protein synthesis and for respiration.

Yemm (1935) showed that starch, sucrose, fructosan and hexoses decreased in amount in leaves of barley during starvation and suggested that each carbohydrate source may make a separate contribution to the respiratory substrate. Yemm (1937) also showed that there is a utilization of nitrogenous substrates during respiration in starving leaves.

In the second category fall those workers who have investigated the effects of desiccation on the amounts of carbohydrates in leaves. Schrödter and Horn (1922), Molisch (1921), Horn (1923), Ahrns (1924), de Wolff (1926), Vassiliev (1931) and Nightingale and Mitchell (1934) agree in showing that with reduction in water content sucrose increases in amount whilst starch decreases in amount.

The results for hexoses are inconclusive. Schrödter and Horn, de Wolff and Vassiliev (*loc. cit.*) reported that no increase in amount of hexoses accompanied decrease in water content whilst Horn, Ahrns and Vassiliev and Vassiliev (1936) found that hexoses, in addition to sucrose, increased in amount when water content was decreased. The last-named investigators have also produced evidence that the relation between starch and sucrose is a reversible one and state that if water is added to a plant in which the starch content has decreased and the sucrose content

increased owing to previous wilting, then on addition of water the amount of sucrose diminishes whilst the amount of starch increases. Vassiliev and Vassiliev (1936) and Clements (1930, 1937a and b) state that hemicelluloses increase in amount during wilting.

Further discussion of the results of the workers enumerated above will be postponed until after our own results have been presented.

DESCRIPTION OF EXPERIMENTS.

Material.

Sorted seeds of Wimmera Rye Grass (*Lolium subulatum*, Vis.) were sown in a mixture of sand and loam on May 6, 1937, and the seedlings transferred to pots containing 3·50 kilograms of washed sand on June 7. The sand was maintained at 70 p.c. of its saturation capacity by addition of distilled water throughout the growing period. The following nutrients were added prior to transplantation of the seedlings:

$Ca(H_2PO_4)_2$	0·3 gm. per pot.
KCl	0·15 „ „ „
$(NH_4)_2SO_4$	0·3 „ „ „
$MgSO_4.7H_2O$	0·15 „ „ „
$FeCl_3$	0·015 „ „ „
$MnSO_4.4H_2O$	0·0003 „ „ „

Three weeks later the seedlings were thinned to four even plants per pot and the following nutrients added:

$Ca(H_2PO_4)_2$	0·3 gm. per pot.
KCl	0·15 „ „ „
$(NH_4)_2SO_4$	0·3 „ „ „
$MgSO_4.7H_2O$	0·3 „ „ „

At intervals of three weeks two subsequent applications were made each of the following:

$(NH_4)_2SO_4$	0·3 gm. per pot.
NaOH	0·06 „ „ „

The pots were placed in the constant environment cabinets and at the same time four pots were harvested and analysed to give values for various components under conditions of the glass house. This treatment has been called the preliminary treatment (P) in the following tables. The temperature in the cabinets was maintained at 24°C. throughout the experiment; the pots were brought up to their correct water content daily and a constant stream of air was kept circulating through the cabinets. By means of sets of shelves at two different heights, two different light intensities were provided in the cabinets. At leaf-level the high light intensity, measured by a calibrated photoelectric cell, was 432 metre-candles; the low light intensity was 342 metre-candles.

Twenty-four hours after the pots were placed in the cabinets the following differential nitrogen treatments were applied:

N—treatment 1.	Treatment A.	0 gm. $(NH_4)_2SO_4$ per pot;	light 432 lux
N—treatment 2.	„ B.	2·00 „ „ „ „ „ „	„ „ „
N—treatment 3.	„ C.	6·00 „ „ „ „ „ „	„ „ „
N—treatment 1.	„ D.	0 „ „ „ „	light 342 lux
N—treatment 2.	„ E.	2·00 „ „ „ „ „ „	„ „ „
N—treatment 3.	„ F.	6·00 „ „ „ „ „ „	„ „ „

Plants were removed for investigation on September 13, 14, 15 and 16; four pots per treatment were removed on each day and spare pots containing plants replaced these in the cabinet in order to maintain the humidity. Results of analyses are given in Table 2.

ANALYTICAL PROCEDURE.

Three plants per pot were removed for analysis, the remaining plant being used for measurement of respiration rate.

In the plants for analysis the leaves were severed at the ligule and weighed in groups corresponding to the pots. The leaves from the four pots corresponding to each treatment were then bulked and cut into lengths ¼ to ½ inch. Two 10 gram samples of the fresh leaves were weighed and dropped into boiling absolute alcohol to inhibit enzyme action. A pinch of $CaCO_3$ was also added to prevent hydrolysis of polysaccharides by organic acids. The remaining material was rapidly dried at 90°C. in an oven through which a rapid stream of air was passing.

The leaf samples in alcohol were boiled under reflux for half an hour. The alcohol was decanted and the residue extracted three times with 70 ml. of 90 p.c. alcohol for 2½ hours each extraction. The extracts were bulked and the alcohol distilled off under as low a pressure as possible. The residue from distillation was taken up with water to a volume of 100 ml. in standard flasks. The residues from the extraction were oven-dried at 95°C.

Aliquots of the aqueous solution, usually 10 ml. each, were cleared by addition of 2 ml. each of 10 p.c. $ZnSO_4$ and 0·5N NaOH and the reducing value estimated by the method of Hagedorn-Jensen. In other aliquots sucrose was hydrolysed by heating for 10 mins. with 2 ml. 0·2N H_2SO_4 on a water bath and the reducing value again determined. Fructose was estimated both before and after hydrolysis by oxidation of the glucose by iodine in alkaline solution according to the method of van der Plank (1936) and then determination of the reducing value by the method of Hagedorn-Jensen. The reducing values so obtained do not give true measures of glucose, fructose and sucrose since a considerable proportion of the reducing value is due to the presence of non-sugar reducing substances.

Estimation of the non-sugar reducing substances is based on the removal of the hexose sugars by fermentation with yeast. A yeast suspension was prepared by washing 40 grams of baker's yeast in a centrifuge until the supernatant liquid was clear; four washings were usually sufficient. The yeast was then suspended in 400 ml. sterile water. Since hydrolysis with acid increases the reducing value of the non-sugar compounds it was necessary to ferment the hexoses in solutions both before and after hydrolysis with acid. 10 ml. of the yeast suspension were centrifuged and 25 ml. of the aqueous extract containing the sugars added. The tube was placed in a water bath at 37°C. for 20 min. and kept well shaken during this time. The yeast was centrifuged off and the glucose equivalent of the reducing capacity of the fermented extract determined. Subtraction of this value from the appropriate total reducing values enables the amounts of glucose, fructose and sucrose to be determined

For the estimation of fructosan, the dried residue from the alcoholic extraction was placed in a water-tight stoppered bottle with 80 ml. water and shaken for four hours in a mechanical shaker. The residue was purposely not ground to a powder but left in the short chaffed strips since grinding causes abrasion of starch grains and renders them partially soluble in cold water (Hanes, 1937). After filtration and washing the filtrate was made up to 200 ml. and aliquots taken in which fructose and glucose were determined by the methods outlined previously. It was found that about 10 p.c. of the total sugar reducing value was due to glucose, a value approximating that found by Archbold (1938a) for the fructosan of cereals. Accordingly only total reducing capacity was determined. Non-sugar reducing substances were present in the cold water extract and these were determined as before after fermentation by yeast. The fructosan content was calculated in terms of fructose.

The residue from the fructosan extraction was boiled with water, cooled and made up to a volume of 50 ml. with water and acetate buffer to pH 4·63. 10 ml. of a solution of β-amylase prepared from barley grains according to the method of Hanes (1936) were added and the tube containing them placed in a water bath at 37°C. for 24 hours. The contents were filtered and maltose estimated in aliquots of the filtrate, after clearing with $ZnSO_4$ and NaOH, by the method of Hagedorn-Jensen.

Hemicellulose was determined in the residue from the starch extraction by boiling the residue under reflux for 6 hours with 2 p.c. H_2SO_4. After filtration the glucose equivalent of the reducing units was determined in aliquots of the neutralized filtrate and taken as a measure of the hemicellulose.

The residue from the H_2SO_4 extraction was well washed to remove all traces of acid, dried and weighed. This constitutes the crude fibre fraction.

Amino–N was determined in the aqueous extract after distillation of the alcohol by the micro-Van Slyke method after clearing. Protein was determined on the dried material by the method described by Barrien and Wood (1939). The pH was detemined on fresh expressed sap by means of a glass electrode. Rate of respiration was determined by the method previously described by Petrie and Wood (1938a).

All analyses were carried out on duplicate samples of material and the mean value obtained. Using the mean dry weights determined from the cut samples and respiration material the analytical results are expressed as percentages of the dry weight. The respiration rate is expressed as mg. CO_2 per gram dry weight per hour. The analytical results are given in Table 2 and the drifts in amounts of the various fractions with time and treatments are illustrated in Figs. 1–6.

THE DRY WEIGHT DATA.

We have examined the dry weight data in some detail in order to determine whether it is a suitable basis for the expression of the analytical results, and also to gain insight into possible material interchanges between plant organs under the conditions of the cabinets.

The unit employed is the mean dry weight of the plant organs per pot, derived from four pots per treatment. Inspection of the data suggested that the mean dry weight was greater under the higher light intensity than under the lower, and therefore we conducted analyses of variance on the high- and low-light material separately. Subsequently it was shown that these differences were not significant, but separation of the two series brought to light effects within the high light series which otherwise might have been obscured.

The value for one treatment under high light is missing for the fourth day, and we have fitted constants and applied analyses of variance: in the low light series we have neglected the single value for the fourth day. The mean dry weights per treatment together with results of analyses are given in Table 1.

In all cases, except that of the leaves under the high light, the effects of time, treatment and interaction are not significantly greater than the variation within classes.

In the leaves under the high light intensity, however, the effect of treatment is significant, whilst time and interaction are insignificant. Since the effect of

treatment is to increase the organic nitrogen within the leaves, we have attempted to eliminate this effect by multiplying the value of protein- + amino-nitrogen for each treatment by 6·25 and deducting the appropriate value from each dry weight determination. The mean values are given in Table 1. Analysis of these data showed that treatment was no longer significant; time and interaction were also insignificant. The mean value for each treatment tends to increase with time although these cannot be shown to be significant.

TABLE 1.

Mean Dry Weights.

		Treatment.					
		High Light.			Low Light.		
Material	*Day.*	A	B	C	D	E	F
Leaves (total D.W.)	Prelim.	4·55	—	—			
	1	4·17	4·33	4·84	4·28	4·09	4·12
	2	4·44	4·73	4·55	3·91	4·41	4·38
	3	4·84	4·92	5·05	4·08	4·71	4·55
	4	—	5·16	4·90	—	4·84	—
		Treatment significant, time and interaction insignificant. Standard error of mean of 4 pots = 0·227. Coefficient of variation = 4·8 p.c.			Treatment, time and interaction insignificant. Standard error of mean = 0·248. Coefficient of variation = 5·7 p.c.		
Leaves (total D.W. minus protein)	1	3·52	3·52	3·94			
	2	3·74	3·84	3·71			
	3	4·14	3·92	4·12			
	4	—	4·17	3·88			
		Treatment, time and interactions insignificant. Standard error of mean of 4 pots = ·227. Coefficient of variation = 5·8 p.c.			Not determined.		
Stems	Prelim.	3·68	—	—			
	1	2·72	2·61	2·89	2·87	3·14	2·46
	2	2·73	2·72	2·78	2·51	2·73	2·92
	3	3·45	3·21	3·19	2·65	4·31	2·25
	4	—	3·79	2·93	—	3·19	—
		Treatment, time and interactions insignificant. Standard error of mean of 4 pots = 0·41. Coefficient of variation = 13·7 p.c.			Treatment, time and interaction insignificant. Standard error of mean of 4 pots = 0·36. Coefficient of variation = 12·5 p.c.		
Roots	Prelim.	3·05	—	—			
	1	3·00	3·89	3·90	3·33	2·83	3·65
	2	3·71	4·41	4·04	3·89	3·84	3·69
	3	3·34	3·05	2·77	2·68	2·81	3·15
	4	—	2·74	2·72	—	3·31	—
		Treatment, time and interactions insignificant. Standard error of mean of 4 pots = 0·54. Coefficient of variation = 15·1 p.c.			Treatment, time and interaction insignificant. Standard error of mean of 4 pots = 0·37. Coefficient of variation = 11·1 p.c.		

The effect of treatment in leaves under low light fails to reach significance, and the analytical data show that under high light synthesis of organic nitrogen compounds was greater than under the low light. This was possibly due to increased absorption of ammonium salt under the lower light intensity: transpiration was presumably less under the lower light since the water content of the leaves was invariably higher under low light than in the corresponding treatment under the high light.

TABLE 2.

Analytical Results.

Preliminary	
Water	521
Protein–N	2·67
Total amino–N	n.d.
Sucrose	11·89
Fructosan	3·56
Hemicellulose	11·53
Starch	0·84
Glucose	1·59
Fructose	nil
Crude fibre	n.d.
Respiration rate	n.d.
pH	6·42

	Treatment.					
Day 1	A.	B.	C.	D.	E.	F.
Water	610	627	512	643	652	673
Protein–N	2·39	2·77	2·49	2·50	2·68	2·74
Total amino–N	0·103	0·220	0·492	0·097	0·183	0·219
Sucrose	9·69	6·44	7·39	4·89	3·83	3·69
Fructosan	2·46	1·25	3·62	3·02	1·08	0·97
Hemicellulose	13·29	12·85	11·81	13·18	12·93	12·83
Starch	0·75	0·66	0·33	0·59	0·61	0·51
Glucose	1·55	1·13	1·65	0·90	1·11	1·09
Fructose	1·26	1·78	1·69	2·54	2·46	1·68
Crude fibre	0·373	0·369	0·432	0·371	0·375	0·372
Respiration rate	2·00	1·76	3·03	2·20	2·10	2·90
pH	5·72	5·65	5·48	5·72	5·63	5·48
Day 2						
Water	637	627	538	690	676	637
Protein–N	2·46	2·87	2·69	2·53	2·69	2·67
Total amino–N	0·093	0·164	0·271	0·130	0·141	0·181
Sucrose	6·13	5·30	5·60	3·85	3·04	3·85
Fructosan	1·81	1·30	0·96	0·70	0·52	1·34
Hemicellulose	13·45	12·14	10·86	13·12	12·65	11·95
Starch	0·59	0·93	0·62	0·68	0·56	0·38
Glucose	1·30	1·35	1·55	1·05	0·82	0·26
Fructose	1·25	1·59	1·42	1·58	0·71	2·34
Crude fibre	0·392	0·384	0·442	0·381	0·366	0·363
Respiration rate	2·66	2·41	3·34	3·40	2·32	3·25
pH	5·62	5·73	5·59	5·71	5·75	5·52

TABLE 2 (continued).

	Treatment.					
Day 3	A.	B.	C.	D.	E.	F.
Water	614	636	505	719	652	583
Protein–N	2·21	3·08	2·67	2·40	2·82	2·77
Total amino–N	0·096	0·168	0·272	0·114	0·228	0·380
Sucrose	7·08	4·54	5·33	2·44	3·67	2·78
Fructosan	2·12	0·26	1·00	0·35	0·72	0·26
Hemicellulose	13·58	12·32	11·60	13·12	12·64	11·48
Starch	0·59	0·64	0·52	0·43	0·57	0·42
Glucose	1·35	0·93	1·03	1·15	1·25	0·66
Fructose	1·32	1·51	1·82	1·17	2·09	1·18
Crude fibre	0·387	0·389	0·422	0·379	0·362	0·360
Respiration rate	1·94	1·60	2·14	1·75	1·21	1·75
pH	5·63	5·72	5·55	5·62	5·70	5·52
Day 4						
Water	—	603	546	—	712	—
Protein–N	—	2·93	3·01	—	2·86	—
Total amino–N	—	0·181	0·302	—	0·181	—
Sucrose	—	5·37	4·80	—	2·91	—
Fructosan	—	0·92	0·39	—	0·40	—
Hemicellulose	—	12·29	11·12	—	11·01	—
Starch	—	0·60	0·54	—	0·54	—
Glucose	—	1·55	1·66	—	0·66	—
Fructose	—	1·09	0·80	—	1·53	—
Crude fibre	—	0·395	0·413	—	0·368	—
Respiration rate	—	2·20	2·61	—	1·76	—
pH	—	5·68	5·60	—	5·68	—

Water, carbohydrate and nitrogen fractions in per cent. dry weight.
Respiration rate in mg. CO_2 per gm. per hour.
Crude fibre in gm. per 10 gm. fresh material.

Since our object was to measure changes in amounts of carbohydrates which themselves form a considerable fraction of the total dry weight it seemed possible that expression of the analytical results on a crude fibre basis might give a more accurate picture. The data show that crude fibre content expressed on a dry weight basis fluctuated about a mean value with all treatments. Since the changes in dry weight do not increase significantly with time or treatment there is no advantage in expressing the results on a basis other than dry weight.

The standard errors of the means for roots and stems are much higher than those for the leaves: on this account although no significant changes in dry weight can be shown, it cannot be concluded that no interchange of material has occurred between them.

RELATIONS BETWEEN TOTAL AND RESERVE CARBOHYDRATES, ORGANIC NITROGEN COMPOUNDS AND WATER.

In this section it is proposed to consider the state of the nitrogen and carbohydrate systems in relation to time, and also to investigate the changes which treatment effects on the total amounts of organic nitrogen compounds and the various reserve carbohydrates—sucrose, fructosan, hemicellulose and starch.

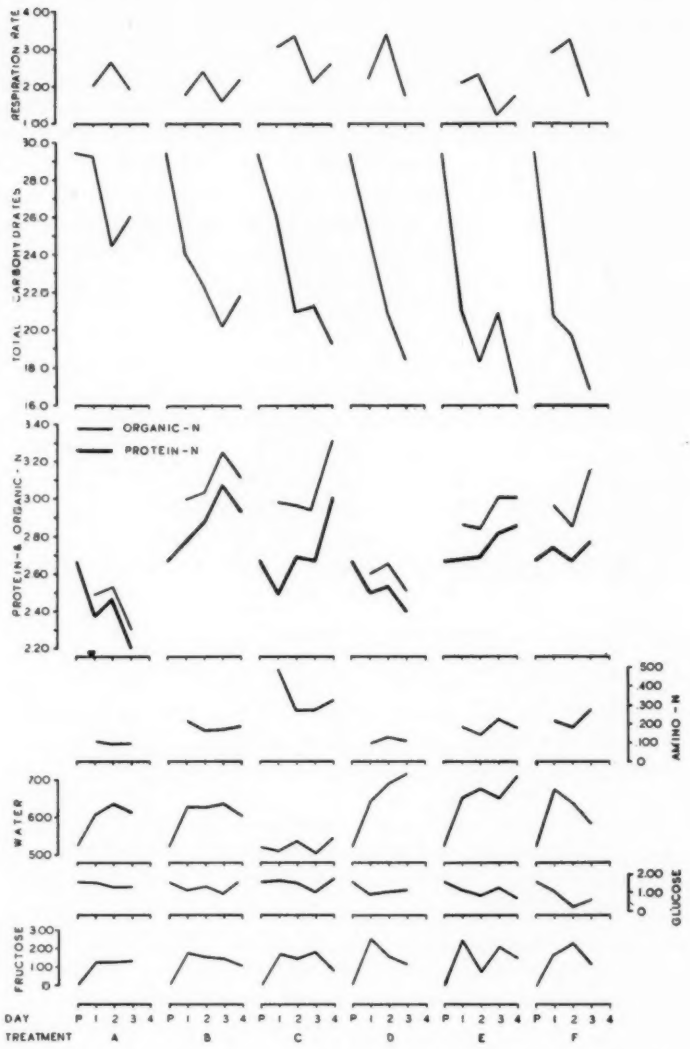


Fig. 1. Drifts with time in contents of various components of the leaves and in respiration rate, all quantities expressed as grams associated with 100 grams dry matter per hour. Respiration rate in mg. CO_2 per gram dry matter per hour.

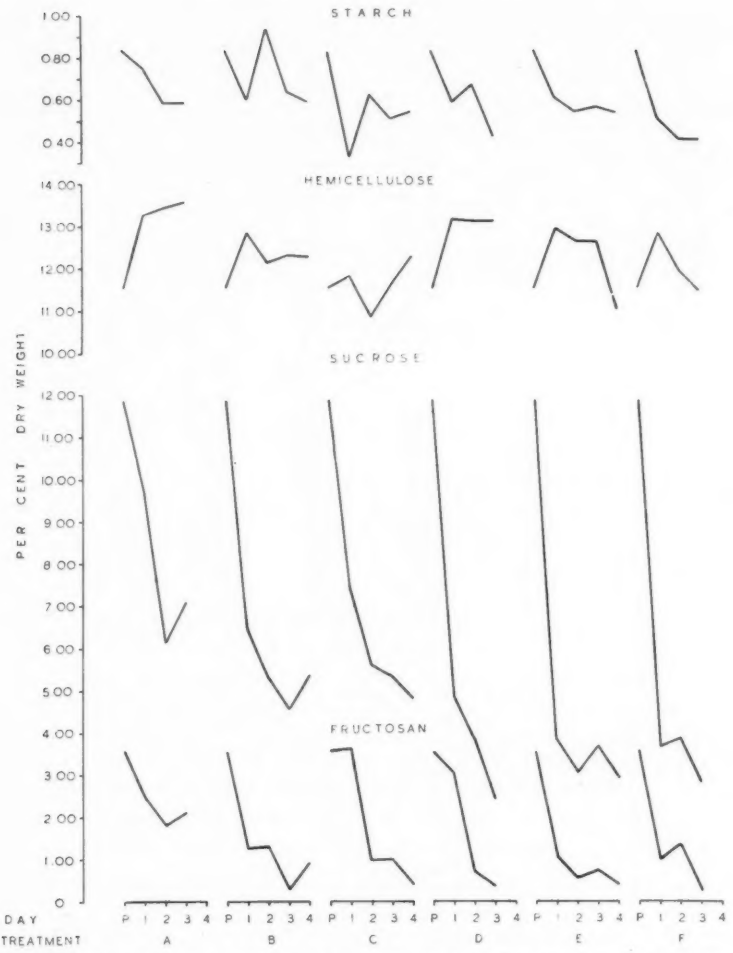


Fig. 2. Drifts with time in contents of reserve carbohydrates in leaves. All quantities expressed as grams associated with 100 grams dry matter.

TIME DRIFTS.

These are plotted for each treatment in Figs. 1 and 2. The water content remained approximately constant during the experimental period under the higher light intensity but shows fluctuations under the low light.

In cases where no extra nitrogen was supplied there is a gradual decrease in amounts of protein-N and in organic-N (measured as protein-N *plus* amino-N).

Such a decrease in control plants is of general occurrence in all our cabinet experiments (*vide* Experiments I, II and III, Petrie and Wood, 1938). On the contrary, where extra nitrogen was supplied protein and organic nitrogen tend to increase in amount.

The amino-N shows little appreciable drift with time under the high light intensity but shows fluctuations under the low light. In general amino-N tends to show a negative correlation with water content.

Total carbohydrates show a marked decrease with time under both high and low light. A similar decrease occurs in the amounts of sucrose and fructosan, the drift curves of these two substances being markedly similar. Hemicelluloses show small fluctuations, but in general a decrease with time is apparent. In the treated plants under high light the amount of starch fluctuates, but shows a decrease after the second day; in all other cases the amount of starch decreases with time.

Comparison of the curves for total carbohydrate, sucrose and fructosan with those for protein-N and organic-N reveals that, except for the untreated plants, the trends for protein-N and organic-nitrogen are in the reserve direction to those for carbohydrates. It cannot be concluded from the time data that increase in organic nitrogen is the cause of the decrease in carbohydrates since it is clear that even in the untreated series the carbohydrates decreased with time. In each treatment series the total carbohydrates decrease with time, but between the series on any one day the total carbohydrate content is lower the higher the organic nitrogen content.

TREATMENT EFFECTS.

Nitrogen Treatment (Figs. 3 and 4). The water content decreases with increased nitrogen treatment, and in general there is a negative correlation between water content and amino-N. As in the experiments described by Petrie and Wood (1938a and b) protein content tends to increase with both water content and amino-N content.

The organic nitrogen (protein-N *plus* amino-N) increases markedly between treatments 1 and 2 under both light intensities, but shows only small variation in amount between treatments 2 and 3. Examination of the experimental data presented by Petrie and Wood (1938a) shows a similar state of affairs. In these experiments the total nitrogen increased with treatment, but if the amount of ammonia-N is deducted from the total-N then it is seen that the amount of organic nitrogen increases considerably with the first treatment of ammonium salt but thereafter varies only to a small degree.

Total carbohydrates decrease markedly between treatments 1 and 2 at both light intensities but exhibit small variations between treatments 2 and 3. The total carbohydrates show a negative correlation with protein-N and with organic-N. ($r = -\cdot 5221$; $\mathbf{P} = 0\cdot 02$.)

Hemicellulose decreases with treatment.

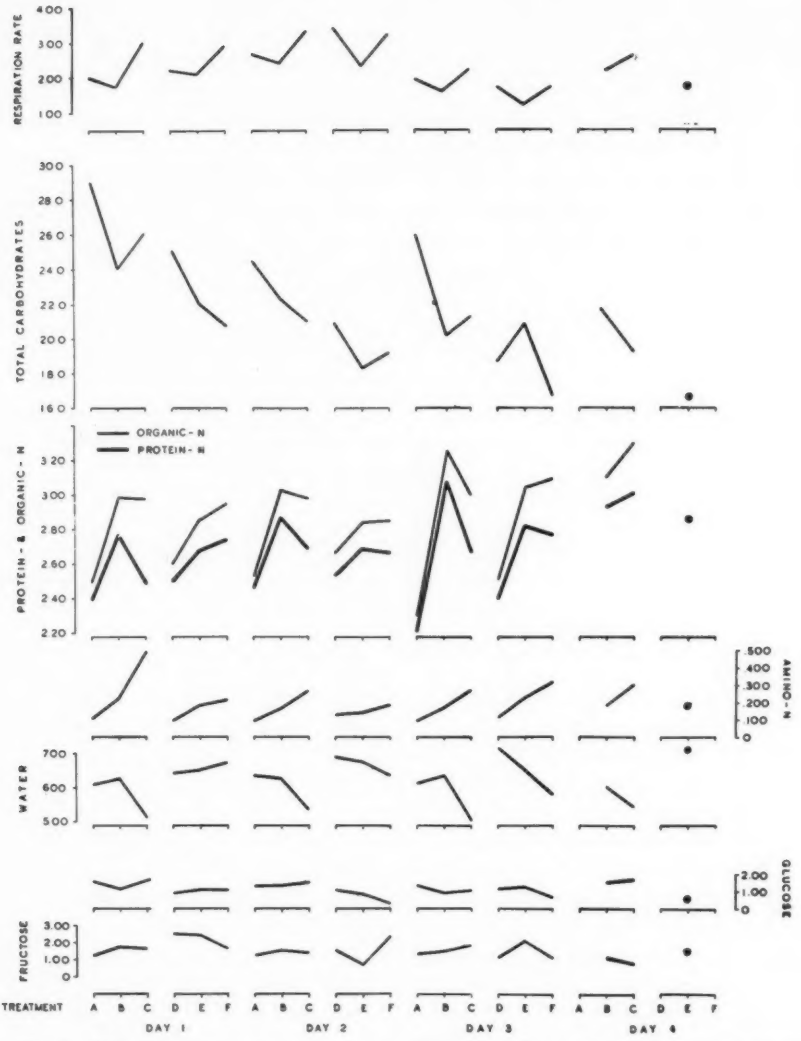


Fig. 3. Effects of nitrogen treatment on contents of various components of the leaves. Units as in legend to Fig. 1.

The starch content shows irregularities probably due to errors in the estimations of the small amounts present, but in general tends to show a decrease between treatments 1 and 3. Sucrose and fructosan show a positive correlation ($r = 0{\cdot}726$;

$P < \cdot 01$). Both decrease in amount between treatments 1 and 2 but increase in amount between treatments 2 and 3. Both sucrose and fructosan show a negative correlation with protein and water contents.

In Figs. 7, 8, 9 and 10 sucrose, fructosan, hemicellulose and starch are plotted against water content, and at each point the value for organic nitrogen content is

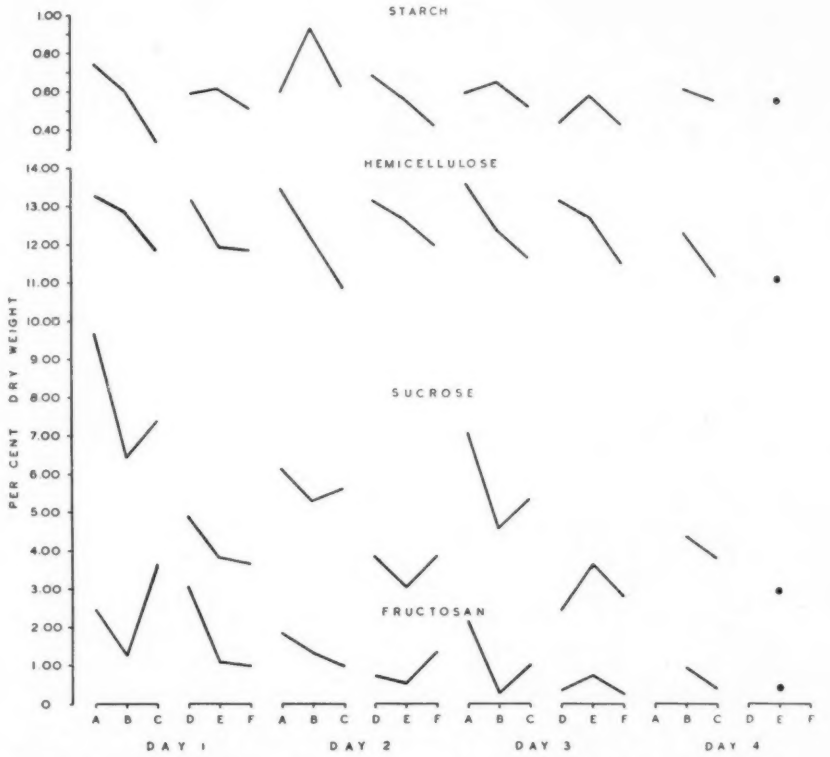


Fig. 4. Effects of nitrogen treatment on contents of reserve carbohydrates in leaves. Units as in legend to Fig. 2.

given. Inspection reveals that sucrose and fructosan tend to decrease with increased organic nitrogen and increase with decreased water contents; hemicellulose tends to decrease with increased organic nitrogen and decreased water contents. The starch data are inconclusive. The values for each day are joined separately and trends with time are apparent.

Light Intensity. These are plotted in Figs. 5 and 6. The total carbohydrates decrease with decreased light intensity at each nitrogen treatment.

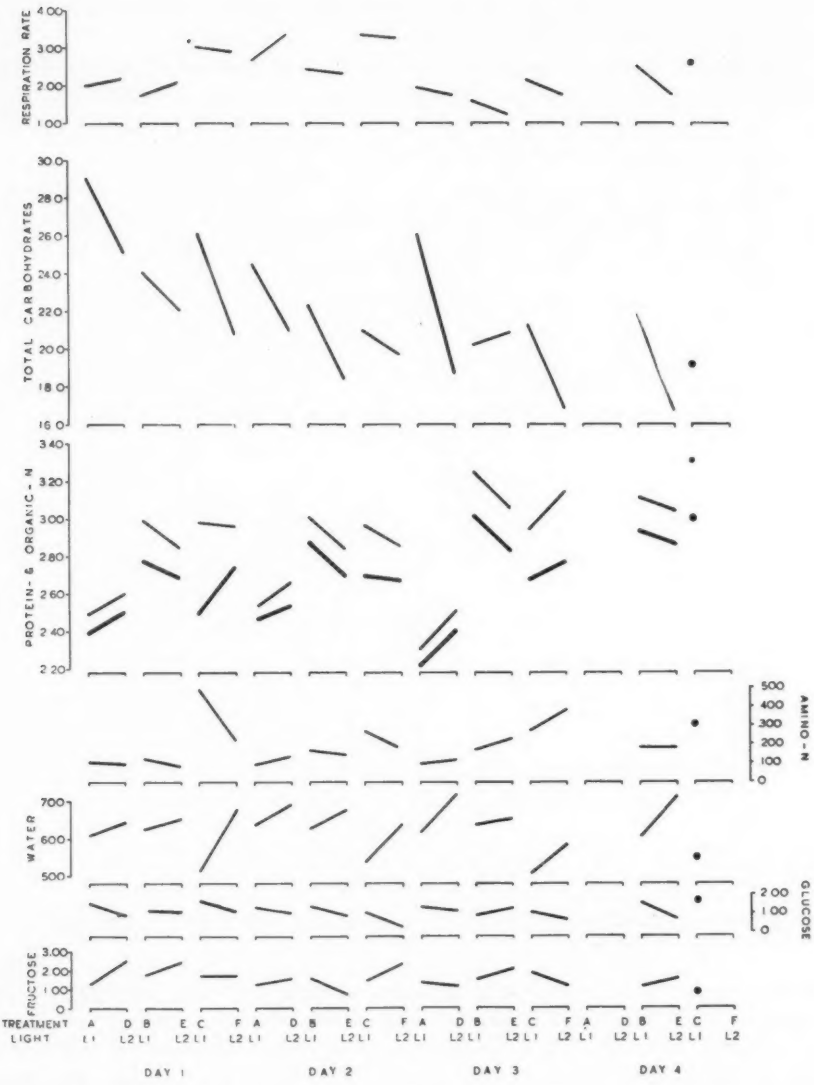


Fig. 5. Effects of light treatment on contents of various components of the leaves. Units as in legend to Fig. 1. L1, high light; L2, low light.

In the plants receiving no extra nitrogen sucrose, fructosan, hemicellulose and starch decrease with decreased light intensity, the decrease being relatively greater in the cases of sucrose and fructosan. In the plants receiving extra doses of nitrogen the effects of decreased light intensity are variable. Sucrose, fructosan

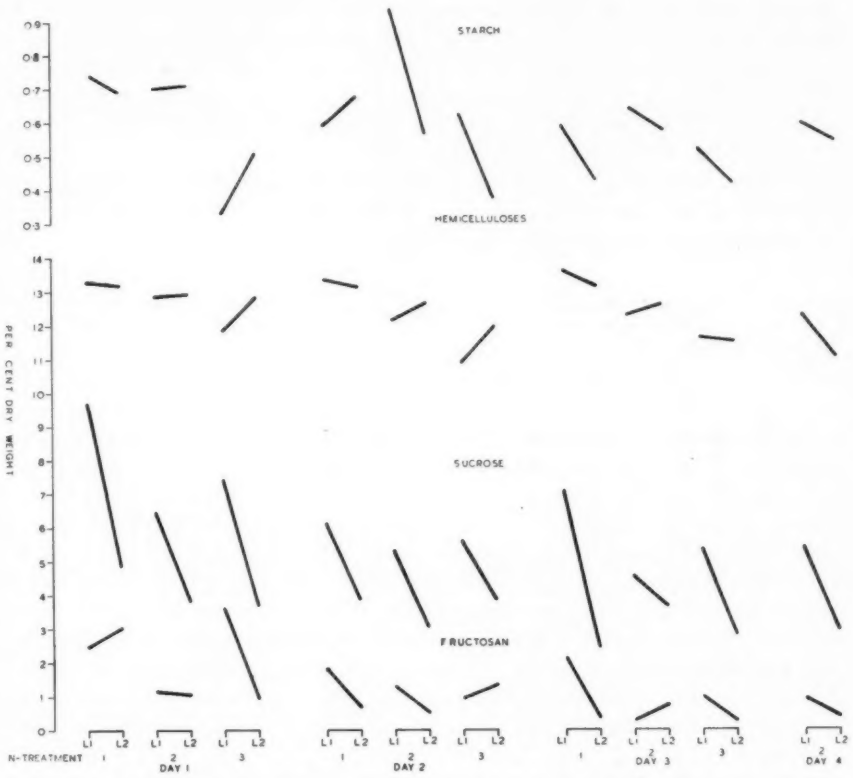


Fig. 6. Effects of light treatment on contents of reserve carbohydrates in leaves. Units as in legend to Fig. 2. L1, high light; L2, low light.

and starch tend to decrease with decrease in light intensity at each nitrogen treatment; whilst hemicellulose increases with decreased light.

Although the carbohydrates might be expected to decrease with decreased light intensity in the untreated plants, it is clear that in the plants receiving extra nitrogen the content of any carbohydrate at any particular nitrogen treatment will depend upon other factors beside light intensity and especially upon the amount of protein synthesized. This is exemplified in the case of hemicellulose. In the

plants receiving no nitrogen the hemicellulose decreases with decreased light intensity, but it cannot be shown that this is due to change in light alone since the organic nitrogen content increases. In all nitrogen treatments the trends for hemicellulose content are in the opposite direction to those for organic nitrogen content. In general the organic nitrogen content is higher in the plants under high light intensity than in those under the lower, consequently there is less utilization of hemicellulose in formation of nitrogen compounds under the lower light.

The same effect of synthesis of nitrogen compounds is noticeable in other carbohydrates, e.g. sucrose, where it can be seen that the decrease in amount of sucrose in passing from high light to low light is greater in those cases where the organic-nitrogen is higher under the low light.

We have calculated the differences between various fractions at high light and low light in the untreated plants and expressed these differences as grams of carbon per 100 grams dry weight as follows:

	Day 1.	Day 2.	Day 3.
Organic nitrogen	0·34	0·34	0·63
Total carbohydrates	2·2	1·4	2·0
Sucrose	1·92	0·9	1·84
Fructosan	0·24	0·44	0·72
Hemicellulose	0·06	0·13	0·18
Starch	0·06	0·04	0·06

All carbohydrate fractions refer to *decrease* in carbon; the organic nitrogen represents an *increase* in carbon. It is apparent that the decrease in amount under low light of total carbohydrates, sucrose and fructosan is greater than can be accounted for by conversion to organic nitrogen compounds, but in the case of hemicellulose and starch this cannot be shown. This does not prove that no decrease in hemicellulose or starch had occurred at the lower light intensity, for if the amount of change of these carbohydrates in response to altered light is small then the effects of organic nitrogen synthesis may obscure the light effect. Possible reasons for the relatively small changes in amounts of hemicellulose and starch compared with those of sucrose and fructosan when any factor is altered will be considered in the section dealing with relations between reserve carbohydrates and hexoses.

Examination of the effect of light suggests that light was limiting and that total carbohydrates and possibly all reserve carbohydrates decreased with decreased light intensity. The data also suggest that all reserve carbohydrates contribute separately to the formation of organic nitrogen compounds.

Statistical Examination. In order to see whether the relations suggested by the time and treatment effects are significant, the data have been examined as a whole by means of partial regressions. The results of these determinations are presented in Table 3.

These show that under both light intensities the total reserve carbohydrates decrease with increased amounts of organic nitrogen (equations 1 and 2, Table 3).

TABLE 3.

Regression Equations.

All regressions given are significant at or below the 5 p.c. point. Coefficients significant at or below the 5 p.c. point are given in italics.

T.R.C. = total reserve carbohydrates; (P + A) total organic nitrogen; Su = sucrose; H = hemicellulose; F = fructosan; Gl = glucose; U = water content. V = percentage of the variance ascribable to the average effect of the independent variables; D.F. = residual degrees of freedom; $\sqrt{\text{res. var.}}$ = square root of variance not accounted for by regression.

				D.F.	V.	$\sqrt{\text{res. var.}}$
(1)	High light	T.R.C.	$= a - b_1(P + A)$ $a = 43{\cdot}095$ $b_1 = \textit{7·765} \pm 1{\cdot}750$	10	65·1	1·782
(2)	Low light	T.R.C.	$= a - b_1(P + A)$ $a = 33{\cdot}677$ $b_1 = \textit{5·731} \pm 2{\cdot}74$	9	51·0	1·867
(3)		Su	$= a - b_1(P + A) - b_2 U$ $a = 27{\cdot}22$ $b_1 = \textit{3·415} \pm 1{\cdot}156$ $b_2 = \textit{0·0201} \pm 0{\cdot}00520$	19	45·84	1·320
(4)		F	$= a - b_1(P + A) - b_2 U$ $a = 12{\cdot}37$ $b_1 = \textit{2·136} \pm 0{\cdot}626$ $b_2 = \textit{0·00805} \pm 0{\cdot}00281$	19	39·74	0·715
(5)		H	$= a - b_1(P + A) + b_2 U$ $a = 15{\cdot}67$ $b_1 = \textit{1·998} \pm 0{\cdot}477$ $b_2 = 0{\cdot}00394 \pm {\cdot}00210$	19	56·98	0·544
(6)		H	$= a - b_1(P + A)$	20	40·00	0·577
(7)			$\frac{Su}{U} \times 10^4 = a + b_1 \frac{Gl}{U} \times 10^5 + b_2 \frac{F}{U} \times 10^5$ $a = 15{\cdot}1$ $b_1 = \textit{0·237} \pm 0{\cdot}0671$ $b_2 = \textit{0·104} \pm 0{\cdot}0287$	19	68·58	19·14
(8)		H	$= a + b_1 \frac{Gl}{U} \times 10^5 + b_2 U$ $a = 5{\cdot}46$ $b_1 = {\cdot}00407 \pm {\cdot}00289$ $b_2 = \textit{·00992} \pm {\cdot}00343$	19	30·71	0·691
(9)		H	$= a + b_2 U$	20	27·14	0·708

The inclusion of a term for water content gave an insignificant coefficient and the value of V was decreased. High values for V were not expected in the regressions described here for a term for respiration which obviously decreases the amounts of the carbohydrates could not be included since measurements of amount of photosynthesis are lacking.

Both sucrose and fructosan gave regression equations of similar form (equations 3 and 4, Table 3) indicating that both carbohydrates decrease with increased organic-N and with increased water content.

The regression for hemicellulose on organic nitrogen and water is given in equation 5, Table 3. Like sucrose and fructosan, the amount of hemicellulose decreases with increased organic nitrogen, but unlike them tends to decrease with decreased water content; the coefficient for the water term just fails to reach significance, but the value of V is decreased by its omission (equation 6, Table 3).

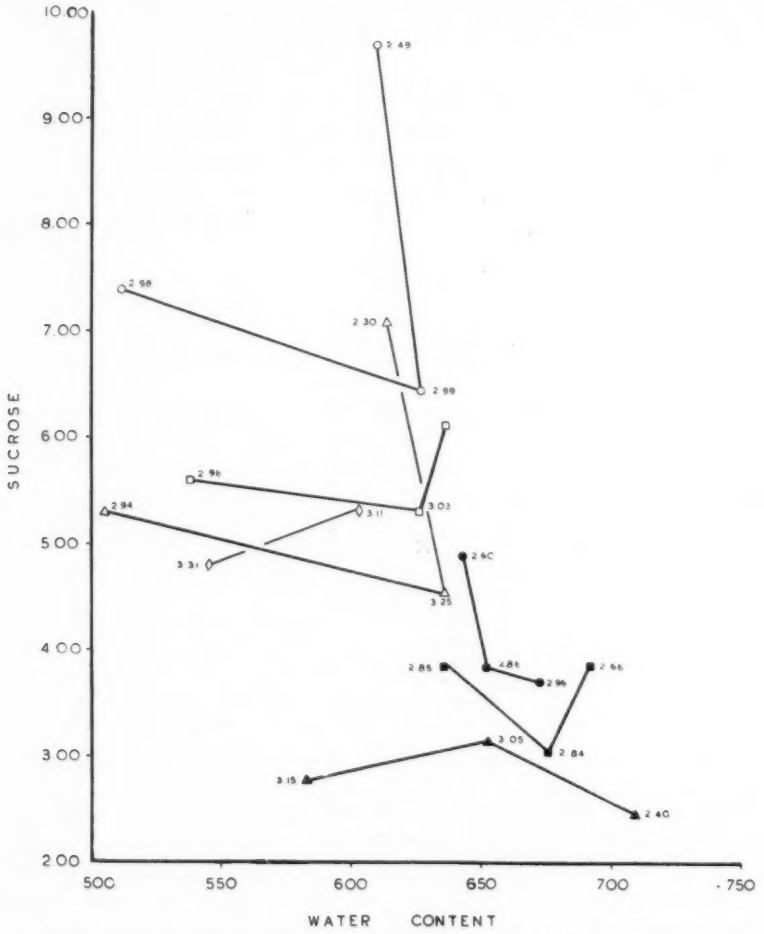


Fig. 7. Sucrose content plotted against water content. At each point the corresponding value of organic nitrogen is given. The symbols marking the points corresponding to the high light treatment have white centres, those corresponding to the low light treatment have dark centres. Symbolism as follows: circles, day 1; squares, day 2; triangles, day 3; diamonds, day 4.

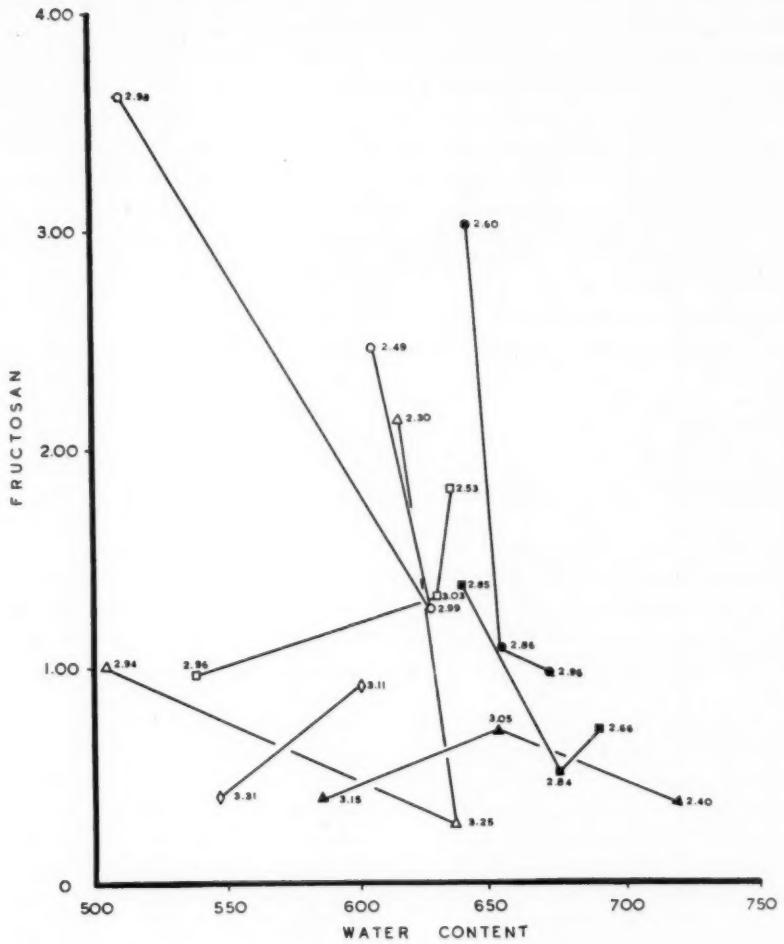


Fig. 8. Fructosan content plotted against water content. At each point the corresponding value of organic nitrogen is given. Symbols as in legend to Fig. 7.

The regression for starch on organic-N and water content did not yield a significant equation. It has been pointed out before that the estimation of starch gave duplicates which agreed badly, and to this we attribute the lack of significance. In considering the position of starch in the ensuing discussions we can rely only on the general trends which are similar to those for hemicellulose,

Conclusions. The picture suggested by examination of the foregoing data is that the system is drifting with time with reference to both nitrogen and carbohydrate cycles.

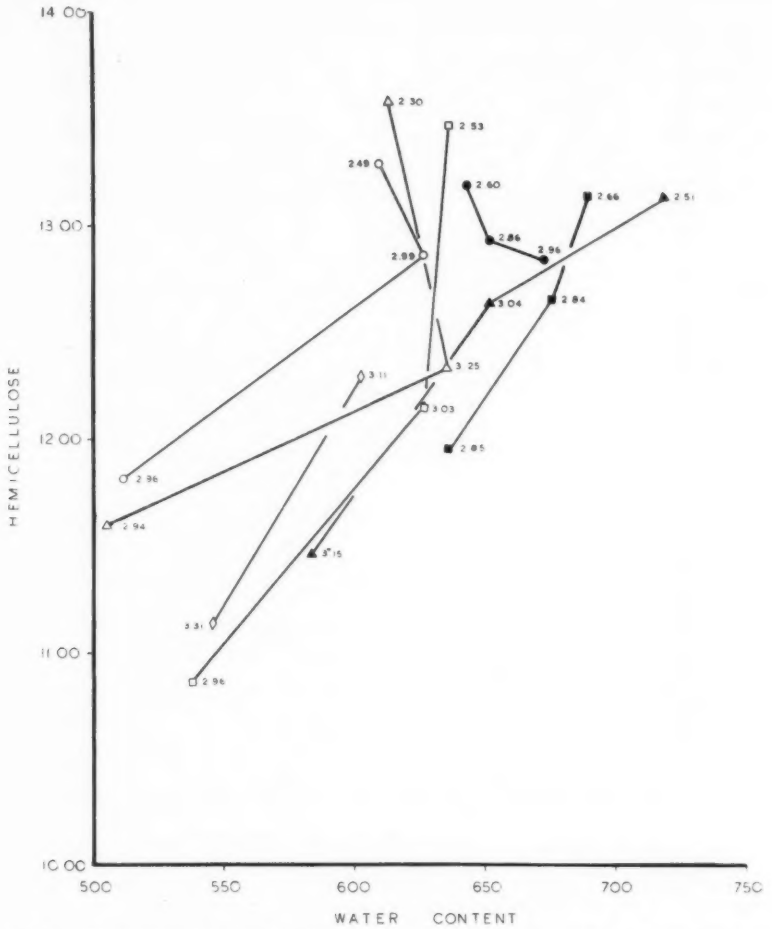


Fig. 9. Hemicellulose content plotted against water content. At each point the corresponding value of organic nitrogen is given. Symbols as in legend to Fig. 7.

The initial decrease in amounts of total and reserve carbohydrates from the preliminary experiment is brought about by transference from the conditions of the greenhouse to the changed conditions of the cabinet, and in the subsequent readjustments translocation may have occurred. The decrease in amount of all

reserve carbohydrates with time over the experimental period suggests that light was limiting and that the rate of respiration exceeds the rate of photosynthesis. Imposed upon this fundamental drift due to excess of rate of respiration over rate of photosynthesis are the effects brought about by increased nitrogen treatment and variations in water content.

The regressions for total reserve carbohydrates on organic nitrogen indicate that the total carbohydrates decrease as the organic nitrogen increases, but that variations in water content are not associated with changes in total carbohydrate content. The regressions for sucrose, fructosan and hemicellulose on organic-nitrogen and water content indicate that in each case the amount of carbohydrate decreases as the organic nitrogen increases. The nature of the water terms in these regressions suggest that hemicellulose (and possibly also starch) decreases in amount as the water content falls, whilst concurrently sucrose and fructosan increase in amount.

The general picture therefore is that each reserve carbohydrate contributes separately to the formation of organic nitrogen compounds, and that with decreasing water content there is a decrease in insoluble reserve carbohydrates accompanied by an increase in the soluble reserve carbohydrates, the total reserve carbohydrate content remaining approximately constant.

The decrease in organic nitrogen with time in the untreated plants may be correlated with decreasing carbohydrates coupled with very low ammonia content, so that synthesis does not occur or may be an expression of ontogenetic drift. The reversal of this normal drift when extra nitrogen is added suggests that although the nitrogen system is drifting it may not be far removed from a steady state and that ammonia concentration was limiting.

The total organic nitrogen in this experiment, as also in Experiments II and III described by Petrie and Wood (1938a), tends to increase in amount with time in treated plants. Also in these experiments the organic nitrogen increases markedly with the first nitrogen treatment but is not appreciably altered by subsequent increased nitrogen treatments. It might be argued that with the high nitrogen treatments extra organic nitrogen compounds are formed, but that any such compounds in excess of the amount in equilibrium with protein are translocated from the leaf. It is apparent from the experiments described by Petrie and Wood (1938a) as well as in this experiment that in general but not invariably, when the protein content decreases with time the amino-acids presumably formed in hydrolysis do not accumulate in the leaves but are translocated therefrom. How far a similar translocation takes place on the same day when different treatments are applied it is difficult to decide. In the present experiment we have attempted to determine this by calculating between the first and successive treatments the increase in carbon due to synthesis of nitrogen compounds (taking 50 p.c. as the proportion of carbon in the organic nitrogen fraction since protein forms the bulk of the fraction), and the decrease in carbon due to loss of total carbohydrates.

Comparing these values and allowing a variation of 5 p.c. in the nitrogen figure for the error of estimation and variability of the material, then the increase in carbon due to organic nitrogen compounds is approximately equal to the loss of

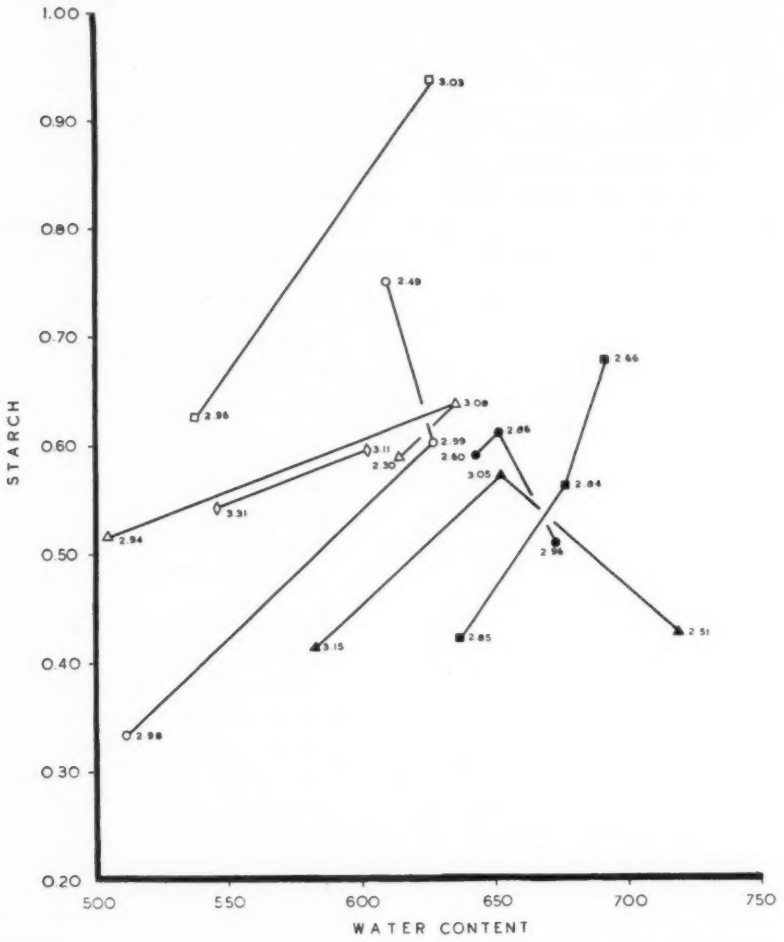


Fig. 10. Starch content plotted against water content. At each point the corresponding value of organic nitrogen is given. Symbols as in legend to Fig. 7.

carbon by the total carbohydrates. It is possible therefore that little translocation of organic nitrogen compounds has occurred from the leaves between treatments on any one day.

Furthermore the data presented by Wood and Petrie (1938) indicate clearly that if the amount of amino-acid-nitrogen or amide-nitrogen is plotted against ammonia-nitrogen the relations are represented by curves concave to the ammonia-nitrogen axis. At low ammonia concentrations the concentration of amino-acid or amide increases rapidly with increase of ammonia, but at higher ammonia concentrations the increase in concentrations of amino-acid or amide is very small with increase of ammonia.

It is generally believed that organic nitrogen compounds such as amino-acids and amides are formed from organic acids derived from glycolytic products or from glycolytic products themselves. The slowing-up or cessation of synthesis of organic nitrogen compounds at high ammonia concentrations is explicable if the amount of carbohydrate substrate in the formation of organic nitrogen compounds (i.e. the rate of formation of glycolytic products) is relatively small and approximately constant and the rate of formation of organic nitrogen compounds is proportional to an intermediate compound formed by the union of ammonia and carbohydrate substrate. The rate of formation of the organic nitrogen compounds would then be proportional to the concentration (i.e. rate of formation) of the substrate except when ammonia was limiting, i.e. present in insufficient quantity to combine with all the substrate.

It is pertinent therefore to enquire into the rate of formation of the substrate for nitrogen synthesis. There are no experimental data for the rate of transformation of hexoses into glycolytic products. However, a special type of rectangular hyperbola is now generally accepted as the form of relation between rate and substrate in many enzymic reactions. In the present case this takes the form

$$R = K.E\frac{S}{S + K_m}$$

where R is the rate of formation of glycolytic products, K is a constant, E is the concentration of enzyme, S is the hexose concentration and K_m is the Michaelis constant.

The form of the curve implies that at higher hexose concentrations the rate of formation of glycolysis products becomes practically constant and practically independent of the hexose concentration. Barker (1936) has shown that in the relatively simple carbohydrate system in the potato stored at low temperatures the relation between rate of respiration and sucrose content is of the form of the Michaelis equation. This may be interpreted as meaning that the rate of respiration is proportional to the rate of glycolysis, which in turn is related to sucrose or the hexose sugars as outlined above.

Such a formulation would also explain why no increase in organic nitrogen content occurs when the water content is decreased and consequently the concentration of all soluble sugars is increased: if the rate of formation of glycolysis products were linearly proportional to the hexose concentration it might be expected that the rate of formation of organic nitrogen compounds would increase

when the water content decreases, but if the rate of formation of glycolysis products conforms to the relation outlined above no such increase in organic nitrogen synthesis would be expected.

The formulation outlined in this section implies that although the rate of formation of organic nitrogen compounds, when the ammonia concentration is high, may be proportional to the carbohydrate substrate, and consequently to other soluble sugars, yet the amount of organic nitrogen present at any time may be unrelated to the carbohydrate content. In other words, there is not a reversible or opposed relation between carbohydrates and nitrogen compounds.

This view is supported by the fact that plants in the dark are capable of forming organic nitrogen compounds if supplied with an external nitrogen source. Furthermore, Gregory and Baptiste (1936) found low protein associated with high sugar content in nitrogen-deficient plants and high protein synthesis associated with low sugar content in potassium-deficient plants. We shall return later to these experiments of Gregory and his co-workers in discussing respiration.

RELATIONS BETWEEN RESERVE SUGARS AND HEXOSES.

The total hexoses on a dry weight basis do not exhibit any discoverable relations to other variables. The same statement is true for fructose, although glucose tends to show a negative correlation with fructose ($r = -\cdot3903$; $\mathbf{P} < \cdot1$, $> \cdot05$).

Attempts to formulate relations between the carbohydrates are difficult since some of the components are in solution in the cell sap as well as in the protoplasm, e.g. hexose, sucrose, fructosan. The partition coefficient existing between the concentration of any one of these components in the vacuolar sap and protoplasm respectively is not known; however, it might be expected that at a steady state the concentrations in the two phases would be proportional to one another. In attempting to discover relations between soluble sugars we have therefore employed concentration terms by dividing the percentage amount of any soluble carbohydrate on a dry weight basis by the percentage amount of water.

Sucrose and Fructosan.

The data expressed on a dry weight basis suggest that the amount of sucrose shows a positive correlation with fructosan and glucose and a negative correlation with water content. The latter affects the sucrose content in two ways: first, by determining the concentration of the reactants, and second, an effect whereby reduction in water content increases the amount of sucrose and other soluble sugars at the expense of hemicellulose. By the use of concentration terms part of the dual effect of water might be expected to be eliminated. The correlation coefficients for sucrose concentration on glucose concentration and on fructosan concentration are both highly significant, the respective values being $r = \cdot713$; $\mathbf{P} < \cdot01$ and $r = 0\cdot726$; $\mathbf{P} < \cdot01$.

At a steady state the relation between sucrose and hexoses would be expressed by the equation

$$[Su]_\epsilon = K_1[Gl]_\epsilon[\gamma Fr]_\epsilon$$

where $[Su]_\epsilon$, $[Gl]_\epsilon$ and $[\gamma Fr]_\epsilon$ represent respectively the equilibrium concentrations of sucrose, glucopyranose and fructofuranose and K_1 is the equilibrium constant. The relation between sucrose concentration and the concentration of the hexoses is not a linear one since the number of molecules is not the same on either side of the equation. If, for example, the equilibrium concentration of sucrose is increased the concentration of the hexoses increases to a less degree, and conversely on dilution the concentration of hexoses decreases in less ratio than that of the sucrose concentration. Over limited portions of the curve, however, the relations between sucrose and hexoses might be approximately linear.

Fructofuranose cannot be estimated separately from fructopyranose by chemical means. Fructopyranose estimated by us in this experiment bears no discoverable relation to sucrose; if, therefore, the system is near a steady state in this experiment the concentration of fructopyranose bears no simple relation to fructofuranose.

The chemical structure of the fructosan present in grasses is not known. However, if it is similar to other inulins and fructosans in plant tissues it is composed of fructofuranose units. As such it might be expected at a steady state to be proportional to the amount of fructofuranose.

On this assumption therefore we have determined the regression of sucrose concentration on glucose concentration and fructosan concentration. The regression is given in equation 7, Table 3. The coefficients for glucose and fructosan concentrations are both significant and the value of V is high. If the system were not far removed from a steady state it would be expected that the percentage of the variance accounted for by the regression should be high. It is probable therefore that although the amount of sucrose may be drifting with time it may not be far removed from the steady state and is correlated positively with both glucose and fructosan.

Hemicellulose and Starch.

Relations between insoluble carbohydrates and soluble sugars are difficult to formulate since heterogeneous systems are concerned and there is lack of knowledge of the mode of enzyme attack upon them. The case of starch may serve as an example.

The action of amylase preparations on native starch derived from storage structures is extremely slow, and microscopic observations indicate that corrosion of the grains begins in localized surface areas. The existence of an external membrane about such grains and resistant to enzymes has been largely recognized. Sande-Bakhuyzen (1935) considers this membrane to be a less hydrated form of

starch. Although such a membrane may be present in starch grains in storage organs where the water content becomes low, it is unlikely to be present in starch grains of the leaf which are temporary structures and where synthesis and degradation of starch are constantly occurring. If this is so, two possible mechanisms for the mode of starch hydrolysis suggest themselves. First, the amylases may attack only "soluble starch". It is difficult to conceive the existence of a Noyes-Whitney layer around the starch grain, i.e. a film containing a saturated solution of starch, since starch in solution consists of aggregates formed by the association of varying numbers of simple macromolecules. If such a layer existed the rate of starch hydrolysis would be proportional to the solubility (i.e. concentration in the saturated layer) and the concentration of the enzyme and be independent of the total starch content.

If the enzyme is capable of attacking the starch grain directly, as microscopic examination suggests, the rate of hydrolysis would be proportional to the surface of the grain exposed and the enzyme concentration.

At any moment there will exist around the starch grain glucose in solution which may have a different concentration from that in the rest of the cell. We may write then

$$+\frac{d\,Gl_f}{dt} = K_1 S.e + K_0([Gl_c] \sim [Gl_f])$$

where $[Gl_f]$ and $[Gl_c]$ represent the concentrations of glucose in the film around the starch grain and in the bulk of the cell respectively, S is the surface of the starch grain and e the amount of enzyme; K_0 is a diffusion constant and K_1 the velocity constant for the conversion of starch into glucose.

The rate of disappearance of glucose around the starch grain is determined by its conversion into starch and its diffusion into the bulk of the cell. The chemical evidence shows that glucose units as they exist in starch have a pyranose configuration, and Hanes (1937) suggests that the simple starch macromolecule contains 30 glucose units. We have then

$$-\frac{d\,Gl_f}{dt} = K_2[Gl_f]^n + K_0([Gl_c] \sim [Gl_f])$$

where n is the number of glucose units in the starch molecule, K_2 is the velocity constant, and the other terms have the same significance as above.

If a steady state is attained the concentration of glucose at the two ends of the diffusion path is equal, i.e. $[Gl_f] = [Gl_c]$, and concentration of glucose in the bulk of the cell is proportional to the surface of the starch grain and the enzyme concentration. The relation between starch and glucose, i.e. $\frac{[Gl]^n}{S.e} = K$, implies that a great change in the surface (i.e. amount) of starch will be accompanied by only a very small change in the glucose concentration.

The above formulation considers only the case where starch and glucose are present in the system. Limitations are imposed when other substances also yielding glucose, as sucrose, are present in the system. In this case the conditions for equilibrium, assuming the amount of enzyme to be constant are:

$$Su = K_a[Gl][\gamma Fr]$$
$$\text{and } St = K_b[Gl]^n$$

Whether sucrose and starch can both coexist in equilibrium with the same glucose concentration will depend on the value of [Gl] and also on the values of the equilibrium constants of the two reactions. The greater is K_b compared with K_a the greater will be range of [Gl] over which the sucrose and starch can coexist in equilibrium with glucose. For very low concentrations of glucose such as occur in our experiments the relation between sucrose and glucose and between starch and glucose may be approximately linear.

Also for low concentrations of glucose it is apparent that a momentary decrease in glucose concentration brought about by a sudden change in the light intensity or by increased protein synthesis requires a relatively large decrease in sucrose concentration and a relatively small decrease in amount of starch in order to attain equilibrium again. This feature is possibly the explanation of the changes in amounts of reserve carbohydrates under low light intensity in our experiments, where it is found that the decrease in amount of sucrose and in fructosan in passing from high light to low light is very much greater than the decrease in amount of hemicellulose and starch.

Similar theoretical considerations apply in the case of hemicellulose as in the case of starch.

The best measures available of the total surface of starch and hemicellulose are the total starch and hemicellulose contents on a dry weight basis, though these must be only approximations since surface will increase to a less degree than volume. To seek for relations existing between starch and hemicellulose and hexose sugars, we have employed as units starch and hemicellulose on a dry weight basis and glucose on a concentration basis.

The amount of hemicellulose falls continuously with treatment, and we have shown in a previous section that the amount of hemicellulose is significantly correlated with organic nitrogen content and with water content. If hemicellulose content is plotted against glucose concentration and for each point the corresponding value of water content is given, it can be seen that the points for low water content (<600) lie below the points for high water content. On the whole data there is a positive correlation for hemicellulose on water ($r = \cdot 485$; $\mathbf{P} = \cdot 05$). The correlation for hemicellulose on glucose concentration is insignificant, but if the values for the low water contents (<600) are removed a significant correlation for hemicellulose on glucose concentration is obtained ($r = \cdot 570$; $\mathbf{P} < 0 \cdot 05$).

We have determined the regression of hemicellulose on water content and glucose concentration (equation 8, Table 3). The coefficient for the water term is significant but that for the glucose concentration is insignificant. However, the removal of the latter term from the regression decreases the value of V (equation 9, Table 3). It is probable therefore that the hemicellulose content decreases with decrease in glucose concentration and with decrease in water content.

Similarly if starch content is plotted against glucose concentration there is suggestion of a linear relation between the variables and the points for low water again tend to fall below those for high water (<600), but significant coefficients for water content and for glucose concentrations were not obtained. It is clear from the literature, however, that decrease in water content causes starch hydrolysis.

The mechanism of the effect of decreased water content in bringing about hydrolysis of hemicellulose and starch is obscure at present. An analogous case is the effect of decrease in water content on the protein-amino-acid equilibrium investigated by Petrie and Wood (1938a). For the carbohydrates probably the simplest explanation at present is to assume that water content increases in some way the value of the equilibrium constant.

The picture which emerges from consideration of the data is that sucrose is positively correlated with glucose and fructosan concentrations, and it is suggested that fructosan content gives a measure of fructofuranose. Hemicellulose, and probably starch, decreases when the water content decreases, and is probably also positively correlated with the glucose concentration. It is possible therefore that the reserve carbohydrates can coexist with a particular glucose concentration and may not be far removed from a steady state.

FRUCTOSE.

The position of fructose in the system investigated by us is peculiar. All other carbohydrates can be demonstrated to involve relationships with one another and with organic nitrogen, but in the case of fructose no significant relation to other variables is discoverable. We have considerable confidence in the values of fructose obtained in this experiment, since fructose as well as total reducing capacity was determined both before and after hydrolysis of sucrose. The mean value of fructose after hydrolysis in any treatment agreed with that obtained before hydrolysis to within 0·5 p.c. in each case.

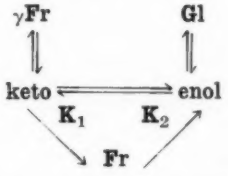
The literature on fructose records various anomalies which suggest that it does not enter into balanced reactions with other carbohydrates. On the preliminary day of the experiment described here the fructose content was nil, although the amounts of sucrose, glucose and fructosan were relatively high. Yemm (1935) in his experiments with starving barley plants has shown that fructose rapidly disappears from the leaves; after from 24 to 72 hours no fructose was found in the leaves although they still contained quantities of sucrose and glucose.

Fructose may also accumulate in quantities greatly in excess of the glucose content in stored fruits (Evans, 1928).

In some cases, e.g., potatoes stored at low temperatures where starch has been converted into sucrose (Barker, 1936) there is a relatively simple system. Here sucrose forms the only soluble ultimate substrate for respiration, and glucose and fructopyranose appear in approximately equivalent quantities as might be expected from the Mass Law if the labile fructofuranose were converted into fructopyranose.

Since the fructose present in the sucrose molecule is in furanose form, and since the amount of sucrose increases at the expense of starch and hemicellulose when the water content is decreased, it is apparent that transformations of the hexose sugars occurs. Furthermore, the labile fructofuranose does not accumulate in plants but is transformed into the pyranose form. There is no evidence at present that the reverse transformation occurs.

It is generally believed that the transformation from fructofuranose to fructopyranose occurs through the formation of the intermediate common keto-form. Further, to explain the interconversion of glucopyranose, fructopyranose and mannose in alkaline solutions and also their common property of being fermented by yeasts, it is assumed that the conversion takes place through the formation of an intermediate enolic form common to the three sugars, and involving an enol-keto transformation. The relations may be expressed in the following scheme:



in which γFr, Gl and Fr represent fructofuranose, glucopyranose and fructopyranose respectively; K_1 and K_2 are the velocity constants for the reactions comprising the formation and disappearance of fructopyranose respectively. Then if K_1 [keto] $>K_2$[Fr] fructopyranose will accumulate in the system and the amount of fructopyranose will depend on the amounts of fructofuranose and of glucose. Fructopyranose may be absent from the system when K_1[keto] $=K_2$[Fr].

In the experiment described in this paper there is a tendency in the majority of treatments for fructose to be higher in the low light treatments than in the high light treatments. In general also the amount of fructose decreases in amount with time. These features of the experiment are explicable on the foregoing scheme if on exposure to the lower light intensity sucrose hydrolyses and rapidly attains equilibrium with fructofuranose and glycopyranose, any hexose in excess of that characterizing the steady state being converted into fructofuranose. The latter then slowly drifts with time towards equilibrium.

RESPIRATION.

Respiration rate as measured by us, using absorption in Reiset towers over 1½-hour intervals and subsequent titration, can at best give an approximate value of the rate at the time of cutting the analytical samples. Since this experiment was performed we have made more accurate estimations of respiration rate on grass leaves using a conductivity method capable of giving measures of the rate over five-minute intervals. These measurements showed that cutting and handling leaves caused an increase in respiration rate followed by a decline similar in character to that described by Audus (1935) and Godwin (1935). In the leaves used by us the rate became steady after about two hours. In the experiment described in this paper the respiration rate became approximately constant after four hours. We have taken the rate of respiration after four hours as a measure of the rate in each treatment.

The data themselves show an abnormality in that the values obtained for the rates for plants on the second day are considerably higher than for corresponding treatments on the other days. We are unable to account for this peculiarity. Its effect has been that we were unable to apply the method of regression. The tendencies exhibited on each day are similar however, and we can only describe these effects graphically.

The most striking feature of the effects of nitrogen treatment on respiration in the experiment described here is the decrease in rate between treatments 1 and 2 and the increase in rate between treatments 2 and 3 on each day and under both light intensities. Generally the decrease in rate is associated with decrease in amounts of reserve carbohydrates and with increase in amount of organic nitrogen; the rise in respiration rate is associated with an increase in amount of sucrose and fructosan and with an increase in amino-nitrogen, and in some cases with decrease in water content.

The decrease in respiration rate between treatments 1 and 2 may be partly determined by decrease in amount of respiratory substrate. It will be recalled, however, that Barker (1936) found that the relations between rate and sucrose approximated to the form of a rectangular hyperbola expected from Michaelis' equation, so that at high sugar concentrations the rate of respiration would show little change with considerable change in sucrose content. Such a view is also supported by the data of Gregory and Sen (1937), who found no change in rate of respiration over a period of 64 hours in barley plants deficient in nitrogen and which contained considerable quantities of sucrose and reducing sugars, whilst the respiration rate declined with time in barley plants deficient in potassium and which contained small amounts of sucrose and of reducing sugars.

It is possible therefore that the decrease in the rate of respiration with the first application of ammonium salt may be due to competition between respiration and organic nitrogen synthesis for a common substrate. In a previous section we

have suggested that the rate of formation of glycolytic products from a carbohydrate substrate is determined by a relation which can be expressed by a rectangular hyperbola. If both respiration and organic nitrogen synthesis utilize glycolytic products, then at a steady state the rate of respiration would be equal to the difference between rates of formation of glycolysis products and rate of formation of organic nitrogen compounds. When the ammonia concentration is very small the rate of formation of organic nitrogen approaches zero and respiration rate becomes nearly equal to the rate of formation of glycolytic products. If ammonia is added to such a system, however, the rate of formation of organic nitrogen increases and consequently respiration rate decreases.

Such a relation between organic nitrogen synthesis and respiration though suggested cannot be substantiated by the present rather inadequate data. Proof of the relation would require continuous measurements of respiration rate during the addition of ammonium salt to plants low in nitrogen and low in sugar contents. After the lapse of a period of time, organic-nitrogen synthesis would obscure such an effect by decreasing the carbohydrate substrate and increasing amino-nitrogen, both of which may then determine the rate of respiration at that time.

Although amount of substrate and organic nitrogen synthesis might account for the decrease in respiration rate between treatments 1 and 2 in this experiment it does not account for the subsequent rise in respiration rate between treatments 2 and 3. Wood and Petrie (1938) in their experiments found that increased respiration rate was associated with decreased water content, and a similar relationship can be detected on each day in the present experiment. Other investigators (Bouillenne-Walrand, 1926; Smith, 1915; Palladin and Sheloumova, 1918; Collorio, 1928) have reported a similar decrease in rate of respiration with decreasing water content.

Decrease in water content may affect respiration in several ways: by increasing the concentration of the carbohydrate substrate; by increasing the amount of soluble carbohydrate by bringing about hydrolysis of insoluble carbohydrates; or by increasing the amount of amino-nitrogen by bringing about hydrolysis of proteins.

The experimental evidence described in the literature for the effect of amino-nitrogen on respiration rate is apparently contradictory. Several observers (Spoehr and McGee, 1923; Genevois, 1927; Schwabe, 1932) have shown that when amino-acids are fed to plants the rate of respiration is increased. In Experiment I described by Wood and Petrie (1938), and apparently also in the experiment described here, respiration rate is determined in part by the amino-acid content. In Experiment II of Wood and Petrie (*loc. cit.*) no significant relation between respiration rate and amino-nitrogen could be found although such was suggested. Gregory and Sen have found in barley plants grown under conditions deficient in nitrogen that the sucrose and reducing sugar content was high and that respiration rate was determined chiefly by the amino-nitrogen content; on the contrary,

in potassium-deficient plants where low carbohydrate was associated with high amino-nitrogen, the respiration rate was determined by the sucrose content. Richards' (1938) work on barley plants grown under varying degrees of phosphorus deficiency shows essentially similar results, namely that in fully-manured plants where high sucrose is associated with low amino-content respiration rate was determined mainly by amino-content, whilst in extreme phosphorus deficiency where low sucrose was associated with high amino-nitrogen the sugar content played an important part in determining respiration rate. Essentially similar results to those of Gregory and Sen and of Richards were found in Experiment III by Wood and Petrie (*loc cit.*). In this experiment water content in two treatments was reduced to a very low level, and associated with the low water content were low sucrose content and high amino-nitrogen content. In Wood and Petrie's paper (1938) values for sucrose were not reported; they are given here together with other pertinent data in Table 4.

TABLE 4.

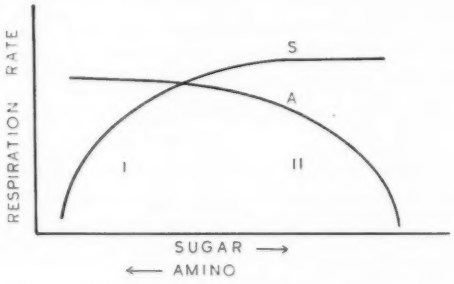
Data of Experiment III, Petrie and Wood (1938a) and Wood and Petrie (1938).

Day.	Treatment.	Water.	Total Amino–N.	Sucrose.	Respiration Rate.
1	C	356	178	6970	3·5
	N1	385	313	5830	4·0
	N2	314	575	4680	3·7
	N3	181	751	2220	2·7
	N4	180	876	1410	2·9
2	C	365	175	7110	3·1
	N1	368	486	4920	3·6
	N2	280	677	3230	3·5
	N3	172	841	1770	2·8
	N4	135	1033	1020	1·8
3	C	373	140	7650	3·5
	N1	354	317	7880	3·9
	N2	314	536	6390	4·3
	N3	192	703	1800	3·0
	N4	164	780	1380	1·8

Water in gm. 100 gm. dry weight.
Amino–N and sucrose in mg. per 100 gm. dry weight.
Respiration rate in mg. CO_2 per gm. per hour.

If respiration rate is plotted against sucrose content either on a dry weight or concentration basis it can be seen that the relation between respiration and sucrose is hyperbolic, and that at high sugar contents the rate is also dependent on amino-nitrogen content, but at low sucrose contents the rate is determined by the sucrose content and apparently is independent of the amino-nitrogen content. The sugar values in Experiment III, as in the experiments of Gregory and Sen (1937) and Richards (1938) cannot be accepted with any great degree of confidence since fermentation was not employed, and they are measures of total reducing substances after hydrolysis rather than of sucrose. It is probable, however, in view of the wide range of values, that they are approximately proportional to the sugars.

In each of the three experiments mentioned high amino-nitrogen content is associated with low sucrose, and low amino-nitrogen is associated with high sugar content. The simplest explanation of the relation of rate of respiration under these conditions appears to be that both sugar and amino-nitrogen compounds act as substrates for respiration, and in both cases the relation between rate and substrate is represented by a hyperbolic function. Alternatively, amino-nitrogen may not itself form a substrate, but as suggested by Wood and Petrie (1938) may activate the enzyme system, the degree of activation increasing with increasing amino-nitrogen until saturation is reached. Diagrammatically, the relations between rate of respiration and sugar and amino-nitrogen in the experiments under discussion could be represented:



In phase I rate of respiration is practically independent of amino-nitrogen content and is determined chiefly by sugar content, whilst in phase II respiration rate is determined chiefly by amino-nitrogen content, and may be practically independent of the sugar.

The above suggestions lack rigid experimental proof, but seem the most likely explanation of the data available at present. Respiration rate might be expected to be determined by the rate of glycolysis, the concentration of amino-nitrogen and by the rate of protein synthesis.

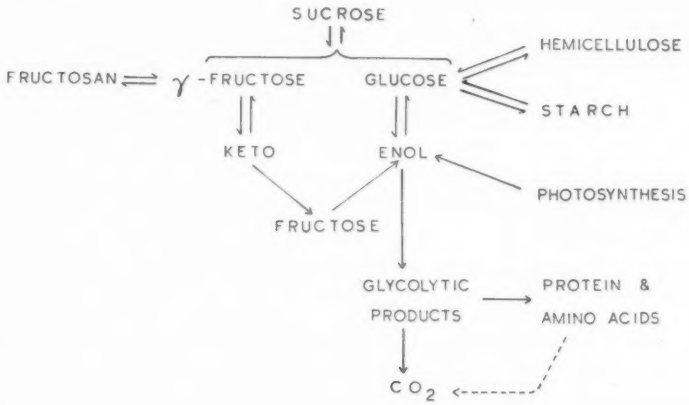
In the present experiment if respiration rate is plotted against sucrose concentration, as the best measure of the enol, points for each day joined together and the value of amino-nitrogen for each point inserted, it can be seen that there is a tendency for respiration to increase slightly with increased sucrose concentration and markedly with amino-nitrogen.

No account has been taken in this discussion of the data of Richards (1938) and of Petrie and Williams (1938) wherein a marked correlation obtains between protein content and respiration rate. No such correlation is apparent in this experiment nor in those described by Wood and Petrie (1938). In the present experiment respiration rate expressed upon a protein basis exhibits the same relations to other variables as on a dry weight basis. It is possible that during the rapid synthesis of protein in our experiment "reserve" as opposed to cytoplasmic proteins are formed. In the present state of knowledge speculation seems unprofitable.

CONCLUSION.

It is obvious that our data are inadequate in several respects, notably for starch and for respiration rate. Nevertheless, the tendencies revealed for these two quantities are in accord with those shown by other workers. It seemed worth while therefore to make a stock-taking at the present time and to consider the possible inter-relations in some detail.

The scheme for the inter-relations between carbohydrates and between carbohydrates and respiration and organic nitrogen synthesis which we have developed in the preceding sections may be expressed:



In the development of this scheme we have approached the problem primarily from a chemical viewpoint, and tried to express the most likely events and the theoretical consequences expected from the formulation in terms of the Law of Mass Action. It has been shown in the preceding sections that the data derived from the present experiment are explicable on such a formulation, and as far as we are aware it also explains the data obtained by other workers.

We have indicated the enol as the first product of photosynthesis, but it is obvious that any carbohydrate or its derivative may be considered as the first formed sugar without altering the relations among the carbohydrates.

One obvious criticism of the experimental work described is that translocation of soluble compounds may have taken place from the leaves. The conditions of the experiment are such that translocation is likely to be reduced to a steady state, and we have adduced evidence in the paper which suggests that translocation between treatments on any one day has not occurred. Furthermore the relations between hexoses and sucrose and hemicellulose are such as would be expected were the system not far removed from a steady state.

SUMMARY.

An experiment is described wherein plants of Wimmera Rye Grass (*Lolium subulatum*, Vis) were grown under identical conditions until the experimental stage, and then placed in an environment constant in certain respects when varying treatments were applied. The treatments consisted of application of three different amounts of ammonium sulphate under two different light intensities. The relations among various carbohydrate fractions, total organic nitrogen, respiration rate and water have been studied and the following conclusions drawn.

(1) Total carbohydrates decreased with time throughout the experimental period, suggesting that rate of respiration was greater than rate of photosynthesis.

(2) Sucrose, fructosan, hemicellulose and probably starch make separate contributions towards the synthesis of organic nitrogen, and decrease in amount as the amount of total organic-nitrogen increases.

(3) Total organic-nitrogen increases in amount with the first application of ammonium salt, but shows only small amount of change with subsequent further additions of ammonium salt.

(4) Evidence is brought forward which suggests that organic nitrogen compounds are not related to carbohydrates in a reversible manner.

(5) When the water content of the leaves is reduced to a low level no change in total carbohydrates occurs, but the amounts of sucrose and fructosan increase whilst the amounts of hemicellulose and possibly also of starch decrease.

(6) The concentration of sucrose is determined by the concentration of glucose and of fructosan. It is suggested that the amount of fructosan may be a measure of fructofuranose.

(7) Hemicellulose content and possibly also starch content are determined by the glucose content and by the water content.

(8) Fructose (fructopyranose) bears no discoverable relation to other carbohydrates.

(9) Values for the rate of respiration are inadequate, but these are apparently determined by the sucrose concentration and by the amino-nitrogen content.

(10) The above results, as well as those of other workers on cognate subjects, are considered in relation to a scheme which is developed in the text.

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THE BIOCHEMICAL CHANGES OCCURRING DURING THE STORAGE OF HUMAN BLOOD

by

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The possibility that stored human blood could be used for transfusion was first realized by Rous and Turner (1916) as a result of their work on the stability of erythrocyte suspensions to which glucose had been added. There are now many reports of the use of stored blood, notably of cadaver blood in the U.S.S.R., the storage of blood for military purposes in Spain, and the "blood banks" established by the Mayo Clinic and by the Cook County Hospital. Only limited information is available of the changes which take place during the storage of blood, and the present paper embodies an attempt to obtain a more precise knowledge of these changes. Workers at the Cook County Hospital have observed that the precentage of reactions after transfusion with blood stored for various periods increases with increase in storage time; and it seemed possible that with more knowledge, conditions might be defined under which blood could be stored longer than has hitherto been possible.

The following biochemical quantities were determined during the storage of the blood: glucose, urea, creatinine, non-protein nitrogen, reduced glutathione and uric acid in whole blood, and the ionic phosphate content of the plasma.

METHODS.

Blood was collected from young healthy individuals into sterile graduated flasks containing 2 p.c. sodium citrate in 0·9 p.c. saline. Except where otherwise stated a volume of blood equal to the volume of the sodium citrate solution was collected. The blood and citrate solutions were mixed by rotation and pipetted aseptically into a series of sterile test tubes fitted with cotton wool plugs protected with washed cotton gauze. Analyses were carried out until the first signs of haemolysis were observed spectroscopically. The tubes were stored together and remained undisturbed until taken for analysis. For analysis a tube was removed from the refrigerator allowed to attain room temperature and carefully mixed by repeated inversion.

Particular attention was paid to the cleanliness of glassware which was heated in commercial sulphuric acid, thoroughly rinsed and boiled in distilled water or steamed, and dried in the oven.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

For the study of blood under anaerobic conditions three parts by volume of blood were collected into one volume of the sodium citrate solution since it was considered desirable to keep the volumes as small as possible. The blood was divided between sterile tubes. To produce anaerobic conditions some of these were placed in larger test tubes containing alkaline hydrosulphite and were alternately evacuated and equilibrated with hydrogen free from oxygen. This process was continued until the oxyhaemoglobin was seen spectroscopically to be reduced. Cultures were made from the anaerobically stored blood and from the aerobic controls, and all were found to be sterile.

For similar experiments with washed cells suspended in saline, containing glucose or sucrose, the blood was collected into sodium citrate containing appropriate amounts of the substance used. The blood was centrifuged aseptically in sterile 90 c.c. centrifuge cups provided with close-fitting aluminium caps. The plasma was pipetted off and the cells washed with saline buffered to pH 7·6 by the addition of disodium hydrogen phosphate or similar saline containing glucose or sucrose. The hydrogen ion concentration of the saline was determined by the glass electrode (tables 5, 6 and 7). After the cells had been washed three times, sufficient of the saline or appropriate solution was added to bring the cell suspension back to the original volume of the blood citrate mixture. The cell suspension was then tubed aseptically and stored in the refrigerator. As no precautions were taken to free the erythrocytes from leucocytes, an erythrocyte suspension in saline was made later. This suspension was obtained by centrifuging the blood lightly and removing the majority of leucocytes in the supernatant plasma. The cells were washed twice in this manner and finally washed as usual. The plasma was obtained free from cells by centrifuging. The first six series of cell suspensions used for analysis were cultured after their period of storage and only one, a member of the first series, was found to be contaminated.

For glucose the method of Hagedorn and Jensen was used. A careful check on the analyses was made by the estimation of the glucose content of an 0·10 p.c. glucose solution at frequent intervals. Where, as described later, glucose was added to the blood, the blood was diluted with distilled water before the estimation. For urea the aeration method originally described by Van Slyke was used, and by careful attention to the blank and the adoption of many of the precautions advised by Kay and Sheehan (1934) it was found possible to obtain accurate results from 1·0 c.c. whole blood. A urease extract from Soy and later from Jack beans prepared as described by Kay and Reid (1934) was used. A suspension of this extract was made in a Clarke pH 7·8 buffer solution. The extract prepared from Jack beans was more efficient and had a smaller blank value than that prepared from Soy beans. The uric acid, creatinine, non-protein nitrogen, and reduced glutathione determinations were carried out in protein free filtrates prepared by the method of Folin and Wu (1919). The precipitation was carried out in the cold and it was always found possible to centrifuge off the precipitated proteins and obtain a perfectly clear filtrate. This centrifugation made the method more economical as regards the quantity of blood used. Creatinine was estimated by the method of Folin and Wu (1919), using 5 c.c. of blood filtrate instead of the 10 c.c. recommended. This was found to give consistent and accurate results. Non-protein nitrogen was determined on 5 c.c. or 7 c.c. of filtrate by micro Kjeldahl. The distillate was collected in N/100 H_2SO_4 and the ammonia liberated by distillation was thus estimated by back titration with N/100 NaOH. The filtrate for uric acid estimation was prepared without laking the erythrocytes, as described by Folin (1930). The method of Benedict and Behre (1930) was at first used but owing to the turbidity of the final solution the arsenotungstic acid colour reagent was discarded for the phosphotungstic acid reagent described by Folin (1933). The iodate titration method of Hess (1924) was used for the determination of reduced glutathione. The phosphate determinations were carried out on a protein-free filtrate obtained by the precipitation of the plasma proteins with trichloracetic acid. The method used was that described by King (1932). All determinations were carried out in duplicate and it was found possible to do a complete analysis on 14 c.c. of whole blood.

RESULTS.

It was found possible to keep the blood for 14–17 days at 0° C. before any sign of haemolysis was observed. The results of analyses carried out over the period of time are shown in Table 1. Except where otherwise stated the final analysis given corresponds to the appearance of haemolysis in the plasma. Haemolysis appears much more quickly (in 8–9 days) when blood is stored at room temperature, but similar changes in the blood constituents appear within this shorter time under these conditions (Table 2). As storage at this temperature effected a considerable saving in the time necessary to complete the survey it was used as far as possible in later experiments.

TABLE 1.

Blood Stored at 0° C.

	Specimen 1.	Specimen 2.		Specimen 3.		Specimen 4.	
Days of Storage.	Glucose.	Glucose.	Non-protein nitrogen.	Urea.	Non-protein nitrogen.	Urea.	Non-protein nitrogen.
0	88	116	44	26	35	17	38
3	75	—	—	26	37	—	—
4	—	85	—	—	—	—	—
5	63	—	48	—	—	18	42
7	42	—	—	—	—	—	—
8	—	76	—	26	44	18	44
10	36	—	—	—	—	—	—
11	—	46	53	26	48	—	—
12	30	—	—	—	—	21	58
14	3	—	—	—	—	—	—
17	2	—	—	—	—	—	—
18	—	8	59	27	55	—	—

Results given in mg. per 100 c.c. blood to the nearest integer.

The ionic phosphate results are given in mg. phosphorus per 100 c.c. plasma.

This table shows the decrease in glucose, the small change in urea and the increase in non-protein nitrogen. In Specimens 1 and 4 there was a trace of haemolysis at the end of fourteen days; Specimen 1 showed definite haemolysis after seventeen days, and Specimens 2 and 3 after eighteen days.

In blood stored either at 0° C. or at room temperature numerous biochemical changes have occurred in the system before any haemolysis is observed. The glucose content of this system invariably decreases almost to zero before haemolysis begins (Fig. 1). The rise in creatinine and non-protein nitrogen is of the same order, 35 p.c.–60 p.c. The rise in urea is much less striking, 10–30 p.c., and that in uric acid is large relative to its original content in the system (Fig. 2). The increase in non-protein nitrogen cannot be accounted for by the rise in urea, creatinine and uric acid (Fig. 3). There is a large increase in the inorganic phosphate content of the plasma (Fig. 2), also an increase of reduced glutathione (Fig. 1).

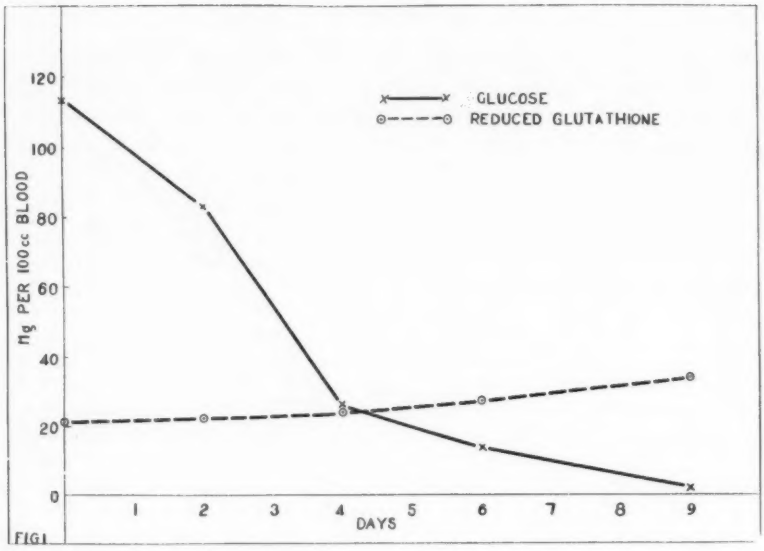

Fig. 1. Aerobic storage of blood. Changes in glucose and reduced glutathione.

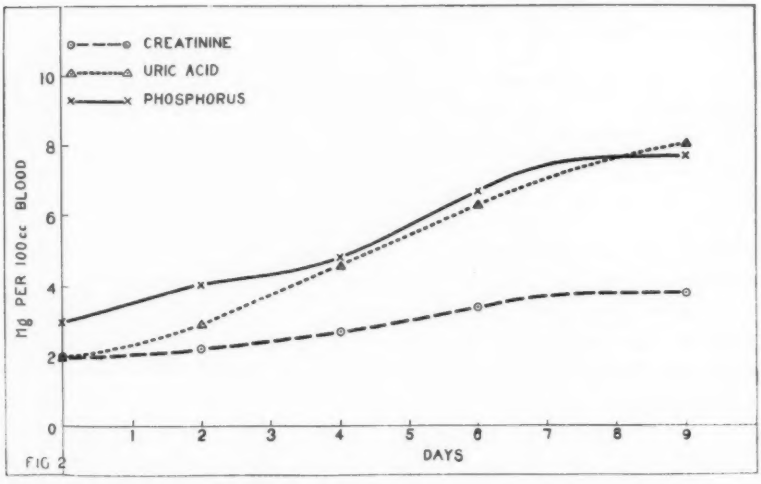

Fig. 2. Aerobic storage of blood. Changes in creatinine and uric acid in the blood and in the phosphate ion in the plasma.

The behaviour of blood stored under anaerobic conditions at room temperature was also investigated. The results obtained are given in Table 3, and show that anaerobic conditions hasten the decrease of glucose but are without effect on the development of the other changes observed.

TABLE 2.

Blood Stored at Room Temperature.

	Specimen 1.					Specimen 2.							
Days of Storage.	Temperature °C.	Glucose.	Urea.	Creatinine.	Non-protein nitrogen.	Temperature °C.	Glucose.	Reduced glutathione.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Phosphorus.
0	22·0	153	25	1·2	45	18·0	113	21	13	2·0	2·0	24	3·0
1	22·5	90	—	1·5	—	—	—	—	—	—	—	—	—
2	—	55	26	1·6	47	17·5	83	22	13	2·2	2·9	30	4·0
3	21·7	29	26	1·8	49	—	—	—	—	—	—	—	—
4	22·0	26	26	2·0	53	18·8	26	24	14	2·7	4·6	41	4·8
5	23·2	23	26	2·0	58	—	—	—	—	—	—	—	—
6	26·0	17	26	2·1	59	16·8	14	27	14	3·4	6·3	51	6·7
7	—	—	—	—	—	—	—	—	—	—	—	—	—
8	—	2	33	2·2	61	—	—	—	—	—	—	—	—
9	—	—	—	—	—	18·6	2	34	15	3·8	8·0	56	7·7

Results given in mg. per 100 c.c.

These specimens show the typical decrease of glucose, small change in urea, and increase of creatinine, reduced glutathione, uric acid, non-protein nitrogen and phosphorus. The results obtained from Specimen 2 are shown in Figures 1, 2 and 3. Specimen 1 showed a trace of haemolysis after eight days and Specimen 2 after nine days.

TABLE 3.

Blood Stored under Aerobic and Anaerobic Conditions.

		Glucose.		Urea.		Creatinine.		Non-protein nitrogen.	
Days of Storage.	Temperature °C.	Aerobic.	Anaerobic.	Aerobic.	Anaerobic.	Aerobic.	Anaerobic.	Aerobic.	Anaerobic.
0	19·6	76	76	23	23	1·9	1·9	38	38
2	21·3	—	22	—	23	—	2·4	—	44
3	20·7	67	—	23	—	2·4	—	44	—
4	19·1	—	22	—	24	—	2·5	—	48
5	21·3	30	—	24	—	2·6	—	50	—
6	22·5	—	19	—	24	—	2·8	—	50
7	22·7	25	—	25	—	2·6	—	54	—
8	22·0	—	2	—	25	—	2·8	—	57
9	22·7	2	—	26	—	2·7	—	60	—

Results given in mg. per 100 c.c.

The table shows the absence of any significant effect on the part of oxygen on the haemolysis of erythrocytes. The first trace of haemolysis was observed on the eighth day in the blood stored without oxygen and on the ninth day in the presence of oxygen.

As the disappearance of the glucose coincided with the appearance of haemolysis it was thought that the addition of glucose might delay haemolysis even at room temperature. Glucose was added to the anti-coagulant and the solution sterilized by filtration. The results are given in Table 4. The cells remained intact up to 15 days, a longer period than had hitherto been observed for storage at this temperature. The optimum amount of glucose appears to be from 0·6–0·7 p.c.

TABLE 4.

The Addition of Glucose.

Days of Storage.	Temperature °C.	Glucose (mg. per 100 c.c.) Control Blood.	Blood + 0·4 p.c. Glucose.	Blood + 0·8 p.c. Glucose.
0	19·6	100	412	880
2	20·0	72	338	726
4	18·2	59	270	662
6	20·6	18	202	500
8	19·9	12	158	426
10	21·0	3	136	392
12	20·2	—	8	370
15	—	—	—	202

The cells of the blood to which 0·8 p.c. glucose had been added showed the first traces of haemolysis on the fifteenth day. The control blood haemolysed on the tenth day and the blood +0·4 p.c. glucose on the twelfth day. The addition of glucose to blood thus delays the onset of haemolysis and the optimum amount of glucose appears to be 0·6–0·7 p.c.

TABLE 5.

The Haemolysis of Washed Cells.

	Cells washed and stored at 0°C. with 0·9 p.c. saline buffered to pH 7·5.					Cells washed and stored at 0°C. with 1·5 p.c. glucose in 0·9 p.c. saline buffered to pH 7·5.				
Days of Storage.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.
0	13	4	0·5	0	13	1330	4	0·5	0	11
5	9	3	0·6	0·4	25	816	4	0·6	0·3	24
11	0	7	1·0	0·6	35	139	6	0·9	0·6	39

Results given in mg. per 100 c.c. cell suspension.

Definite haemolysis occurs with washed cells after five days. It is also found that the non-protein nitrogen increases more readily than in the case of whole blood.

The results obtained for the washed cell suspensions are found in Table 5. This table shows that erythrocytes suspended in saline are very unstable, almost complete haemolysis occurring in five days at 0° C., and that the addition of glucose has no effect on the stability of the cell. The same biochemical changes are observed

as with whole blood, but the increase in non-protein nitrogen is much larger (100–200 p.c.) and is more rapid. The absence of leucocytes does not modify the stability of the erythrocytes or the changes observed in the constituents of the cell suspensions (Table 6).

TABLE 6.

The Haemolysis of Washed Erythrocytes.

	Erythrocytes washed and stored at 0°C. in 0·9 p.c. saline buffered to pH 7·6.							Erythrocytes washed and stored at 0°C. in 1·0 p.c. glucose in 0·9 p.c. saline buffered to pH 7·5.						
Days of Storage.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Reduced glutathione.	Phosphorus.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Reduced glutathione.	Phosphorus.
0	30	6	0·4	0·3	8	15	32	1020	6	0·6	0·3	12	16	30
2	14	6	1·2	2·0	14	17	32	940	6	1·1	2·4	19	17	32
5	3	7	2·2	3·7	16	20	33	832	7	2·4	3·5	21	19	34
7	3	7	2·8	4·3	20	19	34	—	7	2·7	4·9	22	22	35
9	0	7	3·2	5·4	23	26	36	745	7	3·8	6·1	25	29	36

Results, except for phosphorus, given in mg. per 100 c.c. cell suspension. The concentration of phosphate ion is expressed in mg. phosphorus per 100 c.c. of the original volume of the plasma.

The absence of leucocytes does not modify the effects of storage, but the uric acid increase is larger and more rapid than takes place in the presence of leucocytes.

It has been shown that erythrocytes suspended in saline can be agglutinated by the addition of zinc sulphate, and as long as the cells remain intact the haemoglobin cannot be precipitated nor are its essential properties in any way impaired.[2]

The agglutinated cells show biochemical changes similar to their normal controls, but the erythrocytes remain intact for 4–5 weeks, at the end of which time almost complete haemolysis occurs. The glucose in the system disappears 3–4 weeks before haemolysis occurs, and the addition of glucose causes no further delay in the onset of haemolysis.

The biochemical changes that occur during storage in the presence of sucrose were also studied. The erythrocytes were washed and suspended in a solution of 8 p.c. sucrose in 0·9 p.c. saline, the sodium chloride preventing the agglutination which is otherwise known to occur. The non-protein nitrogen increase is much less than that which takes place with normal washed cells, or in whole blood (Table 7). The stability of the cells in the presence of sucrose is most striking, the erythrocytes remaining intact for 4–5 weeks before showing any signs of haemolysis. With whole blood the inhibiting effect of the sucrose is not so well marked (Table 8), nor is there the striking increase in the stability of the cell which is found in the cell suspension stored in the presence of sucrose. The non-protein nitrogen increase which takes place under varying conditions of storage is shown in Fig. 4.

[2] To 120 c.c. of cell suspension were added 4 c.c. M/20 Zn SO_4.

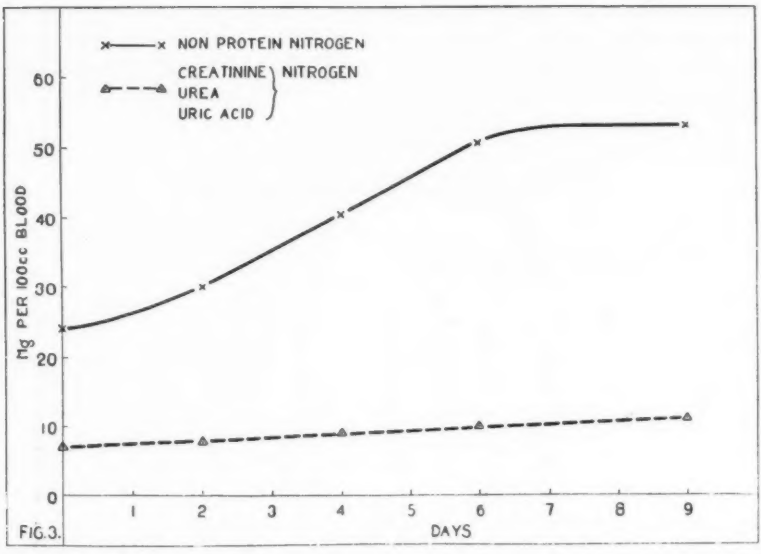

Fig. 3. Aerobic storage of blood. Changes in the non-protein nitrogen and in the combined creatinine, urea and uric acid nitrogen.

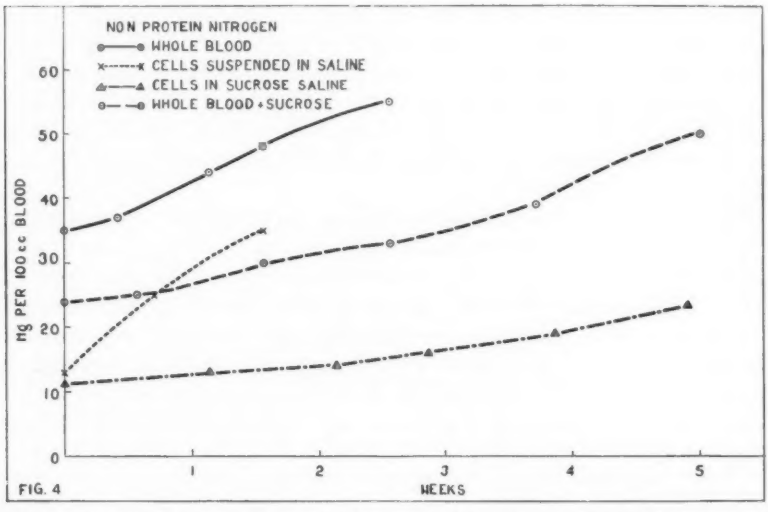

Fig. 4. Aerobic storage. Changes in the non-protein nitrogen of whole blood, of whole blood with added sucrose, of washed cells and of washed cells with added sucrose.

TABLE 7.

The Effect of Sucrose.

Days of Storage.	Cells washed and stored at 0°C. with 8 p.c. sucrose in 0·9 p.c. saline buffered to pH 7·5. Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Reduced glutathione.	Phosphorus.	Control cells washed and stored at 0°C. in saline buffered to pH 7·6. Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Reduced glutathione.	Phosphorus.
Specimen 1.												
0	3	0·4	0	11	19	32	4	0·4	0	11	16	33
8	3	0·5	0·3	13	19	35	5	0·4	0·3	24	18	36
15	4	0·5	0·4	14	20	38	—	—	—	—	—	—
20	5	0·5	0·5	16	22	39	—	—	—	—	—	—
27	6	0·9	0·6	19	23	42	—	—	—	—	—	—
34	6	1·0	0·6	23	24	43	—	—	—	—	—	—
Specimen 2.												
0	5	0·5	0	10	20	31	5	0·4	0	13	17	32
9	5	0·6	0·3	18	29	32	5	0·9	0·4	32	28	35
20	6	1·5	1·0	24	31	34	5	2·6	2·1	40	36	38
44	7	2·4	1·9	30	39	40	—	—	—	—	—	—

Results except for phosphorus, given in mg. per 100 c.c. cell suspension. The concentration of phosphate ion is expressed in mg. phosphorus per 100 c.c. of the original volume of the plasma.

The cells of Specimen 1 began to haemolyse after 34 days and those of Specimen 2 showed definite haemolysis after 44 days. The cells washed with saline showed considerable haemolysis after eight and nine days respectively. The outstanding features of the results shown in this table are the increased stability of the cells washed with saline containing sucrose and the concomitant delay in the increase of the non-protein nitrogen.

TABLE 8.

The Effect of Sucrose.

Days of Storage.	Whole blood collected and stored at 0°C. in 8 p.c. sucrose in sodium citrate. Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Reduced glutathione.	Phosphorus.
Specimen 1.						
0	15	2·0	1·6	24	18	3·1
4	15	2·0	1·7	25	19	3·7
11	15	2·2	1·9	30	20	4·6
18	15	2·4	2·4	33	20	5·4
26	16	2·5	2·8	39	24	6·7
35	17	2·9	3·0	50	26	8·3
37	17	3·1	4·5	55	27	9·5
Specimen 2.						
0	12	2·0	2·3	26	18	3·8
4	12	2·0	3·0	27	24	4·2
13	13	2·2	3·2	32	25	6·0
15	14	2·5	4·0	39	28	7·3
34	18	3·4	5·7	48	32	9·6

Results given in mg. per 100 c.c.

There was a trace of haemolysis in Specimen 1 on the 26th day and in Specimen 2 on the 30th day. The addition of sucrose to whole blood delays the increase in non-protein nitrogen and uric acid.

The results obtained from a series of analyses carried out on stored plasma are shown in Table 9, and indicate that in the absence of erythrocytes the biochemical changes which occur are relatively small.

TABLE 9.

The Storage of Plasma.

		Plasma stored at room temperature.						Plasma and 1 p.c. glucose stored at room temperature.					
Days of Storage.	Temperature °C.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Phosphorus.	Glucose.	Urea.	Creatinine.	Uric acid.	Non-protein nitrogen.	Phosphorus.
0	20·0	56	13	1·1	2·2	25	3·4	1210	12	1·2	2·9	21	3·6
2	21·0	50	13	1·2	3·5	26	4·0	1110	12	1·2	3·9	23	3·8
4	—	45	14	1·5	5·4	28	4·2	—	13	1·6	4·0	25	3·9
7	19·2	37	14	1·6	5·6	31	4·5	960	13	1·7	4·2	29	4·2

Results given in mg. per 100 c.c. plasma.
In the absence of erythrocytes and leucocytes the biochemical changes that take place are small.

DISCUSSION.

The onset of haemolysis in stored blood appears to be associated with two significant changes in the biochemical composition of the blood. These are an increase in both the non-protein nitrogen of the blood and the ionic phosphate content of the plasma. While the glucose content of the blood has usually fallen to a negligible level before haemolysis sets in, the artificial maintenance of a high concentration of glucose will only delay the onset of the haemolysis for a short period. In this connection it may be remembered that according to Irving and Kay (1926) the blood glucose *in vivo* is almost entirely within the erythrocyte, while glucose added *in vitro* to shed blood remains in the plasma. Cells washed free from plasma with buffered saline, with or without addition of glucose, haemolysed much more readily than did erythrocytes suspended in their own plasma, and the non-protein nitrogen increase was concurrently larger and more rapid. When, however, cells were washed and stored in buffered saline containing 8 p.c. sucrose their stability was markedly increased and the changes in the concentrations of the nitrogenous constituents were slower. As the sucrose was dissolved in saline the erythrocytes were not agglutinated to any extent, so that any effect due to mechanical aggregation of the erythrocytes appears to be excluded.

The very slight increase in the concentration of the phosphate ions in the stored plasma, compared with that observed in whole blood, indicates that the ionic phosphate content has some connection with the haemolytic process. It should also be noted that so far in this work no inhibition of the phosphate increase has been apparent.

SUMMARY.

The storage of human blood is accompanied by a decrease in the glucose content of the blood, an increase in the reduced glutathione, uric acid, creatinine, non-protein nitrogen and inorganic phosphate. There is little increase in the urea content of the blood.

The onset of haemolysis may be delayed by the addition of glucose. The optimum amount of glucose is 0·6 to 0·7 p.c. A small part only of the increase in non-protein nitrogen is accounted for by the increase in urea, creatinine and uric acid. Haemolysis of the erythrocytes generally occurs when the non-protein nitrogen reaches a concentration of 55–60 mg. per 100 c.c. blood.

The biochemical changes that take place may be attributed to the erythrocytes, but the essential nature of the process is unknown.

As the inhibition of haemolysis does not seem possible as yet, it should be stressed that the rigorous cleansing of all glass and purification of reagents must be undertaken to procure consistent and reproducable results in this work.

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